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W.D. Gould and/et S. Lord

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THE ENERGY OF OUR RESOURCES - THE POWER OF OUR IDEAS

L'ÉNERGIE DE NOS RESSOURCES - NOTRE FORCE CRÉATRICE

Proceedings of the Eight Annual General Meeting of BIOMINET

October 31, 1991
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Edited by W.D. Gould and S. Lord

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PROCEEDINGS OF THE EIGHTH ANNUAL GENERAL MEETING OF BIOMINET

FOREWARD

BIOMINET presents in this volume the invited technical papers which were presented by members of BIOMINET at the Eighth Annual General Meeting which was held at the Crossroads Motor Hotel, Calgary, Alberta on October 31, 1991.

The morning session consisted of technical presentations on corrosion problems in the petroleum industry and the biological treatment of petroleum wastes. The afternoon session consisted of a panel discussion on environmental problems in the petroleum industry.

The efforts of those who participated in the panel discussion and the members who presented papers are greatly appreciated.

W.D. Gould
S. Lord
Editors

COMPTE RENDU DE LA HUITIEME RÉUNION GÉNÉRALE ANNUELLE DE BIOMINET

AVANT-PROPOS

Ce volume comprend les exposés techniques présentés par des membres de BIOMINET lors de la huitième réunion annuelle, tenue le 31 Octobre 1991 à Calgary, Alberta, au Crossroads Motor Hotel.

La séance du matin comprenait les exposés techniques sur les problèmes de corrosion dans l'industrie du pétrole et le traitement biologique des résidus du pétrole. La séance de l'après-midi comprenait une réunion-débat sur les problèmes environnementaux dans l'industrie du pétrole.

Nous apprécions grandement les efforts des participants à la réunion-débat ainsi qu'aux membres qui ont présentés leurs articles.

W.D. Gould
S. Lord
Éditeurs

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FIELD STUDIES OF BIOCORROSION AND ITS CONTROL IN WATER PIPING SYSTEMS

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ABSTRACT

Biologically induced corrosion is a significant problem in oilfield water piping systems requiring extensive and expensive use of biocide formulations. This paper describes findings from research projects carried out in a unique field test facility designed to investigate the sessile microbial populations and deposits associated with corrosion and optimize control protocols.

ÉTUDES SUR LE TERRAIN DE LA BIOCORROSION ET DES MÉTHODES DE LUTTE CONTRE LA BIOCORROSION DES CANALISATIONS D'EAU

RÉSUMÉ

La corrosion d'origine biologique constitue un problème important dans les réseaux de canalisation d'eau des champs pétroliers qui exige l'utilisation d'une grande quantité de préparations dispendieuses de biocides. Cette étude décrit les résultats obtenus dans le cadre de projets de recherche menés dans une installation d'essai sur le terrain unique afin d'examiner les populations microbiennes sessiles et les dépôts associés à la corrosion ainsi que pour optimiser les méthodes de lutte contre ce phénomène.

INTRODUCTION

Surface bound microbial populations rich in sulfate reducing bacteria have long been associated with corrosion problems in oilfield water handling systems (1). In 1987, NOVA HUSKY Research Corporation established a field test facility in collaboration with Husky Oil Operations Ltd. to develop and optimize effective biocide treatment programs in an oilfield operation near Wainwright, Alberta. In this field, residual oil is produced by secondary recovery or waterflooding wherein water is injected to displace petroleum from the underground formation. The produced fluids are separated in a free water knock out and the water phase returned to tanks in a waterplant prior to reinjection into the reservoir to displace more oil.

The field test facility which is located between the water storage tanks and the main injection pump for the field consists of five test spools mounted on a skid. Water is taken from the injection line, pumped through the skid pipe runs and returned to the water injection system. Water flow in the test unit is in the turbulent flow regime and is not recycled to any significant extent. Four of the test spools are equipped with timed biocide injection facilities. Pressure drop data, flow rates and corrosion rates can all be measured in each line and removable studs allow sampling of surface deposits developed on the internal steel surface of the piping.

This paper reviews the nature of the biofouling which is associated with corrosion damage in this facility and presents new observations with respect to the composition of the sessile microbial population, its dependence on water chemistry and the character of iron sulfide precipitates associated with the surface biofilm. This work was supported in part by a research contract from Energy, Mines and Resources Canmet (#23440-1-9062), Dr. Ron McCready, Scientific Authority.

MICROBIAL BIOFILMS AND BIOCIDE CONTROL

Past observations have established the importance of biofouling in these systems. Initially, piping in the test unit displayed a Darcy friction factor consistent with water flow through a clean, smooth pipe. After about eighty five days of produced water flow, a dramatic increase in the resistance to flow corresponding to more than a doubling of the friction factor coincided with the development of surface deposits. Scanning electron microscopy revealed the presence of a mixed bacterial population embedded in these deposits associated with precipitates and debris. Elemental analysis by energy dispersive X-ray analysis was consistent with the involvement of sulfate reducing bacteria in that iron and sulfur were abundant corresponding to the precipitation of iron by bacterially produced sulfide. In some cases, phosphorus was notable in the bulk deposits presumably due to the concentration of phosphates in the embedded biomass. Viable cell counts by the most probable number technique (1) using Postgate's medium B (2) showed that sulfate reducing bacteria represented a significant fraction of the total cells present as established by epifluorescent counts using a fluorescein dye.

This sessile population is a difficult target for biocide control. Of more than twenty commercial products screened in the past only glutaraldehyde and cocodiamine formulations were able to effect a significant kill in this system (a significant kill being a reduction of at least three orders of

magnitude in the density of viable sulfate reducing bacteria colonizing the surface). Even in the best kills, a residual viable population persisted at about a thousand viable cells per square centimeter of steel surface. Sterilization of these piping systems through addition of biocides is well beyond economic practicality. This is troublesome since a quantitative relationship between cell numbers (or any other biological parameter for that matter) and corrosion rates or risk has yet to be discovered. This makes quick cost benefit analysis through simple direct measurements for a biocide treatment impossible leaving industry in the position of applying biocides at the maximum dosage economics permit and assessing long term failure data for evidence of efficacy and quantitative benefit.

Killing the microorganisms embedded in surface deposits does not result in their removal from the steel surface. Even after more than 99.99% of the sulfate reducing bacteria are killed, no significant change is seen in either the Darcy friction factor arising from fouling or in the total sessile cell counts seen for the biofilm.

Previous studies using an upstream ultraviolet sterilization unit on two of the test lines have shown that the influx of free floating planktonic microorganisms carried into the test system from upstream is not an important factor in the further colonization of the steel surface by or the sustenance of an established biofilm. Little difference was seen between lines receiving disinfected water and those receiving an untreated flow of field brine with or without periodic biocide injections over a period of several weeks. Apparently, under the conditions of these tests the main source of microorganisms for surface colonization or for regrowth between biocide slugs was the production of organisms by the biofilm itself and not recruitment of planktonic organisms from the water entering the system from upstream.

In summary, the sessile population at the waterplant which is the target of the studies described in this paper exists as a mixed consortium of organisms dominated by sulfate reducing bacteria embedded in a matrix rich in iron sulfides. Most commercial biocides have proven ineffective in these facilities and once established the biofilm is able to sustain and extend itself irrespective of the influx of fresh organisms from upstream.

SESSILE POPULATIONS AND WATER CHEMISTRY

In order to investigate the relationships between water chemistry and the biofilm microbial population, two types of studies were carried out. In the first, the water chemistry of the produced water flowing through the unit was monitored and correlations were attempted between changes in various water parameters and viable cell counts. In the second, sessile populations developed on bull plugs mounted in water piping in other parts of the oilfield were brought to the test facility and changes in the composition of these foreign populations on exposure in the test facility were monitored.

Water Chemistry

Table 1 shows the initial water chemistry for the two experimental periods used in the first study. The brine present is highly saline and rich in sodium chloride. The unusually low sulfate levels observed are consistent with an excess of barium in these waters (not shown) which results in barite (barium sulfate) precipitation. Variation in the analyses shown in Table 1 was monitored over twelve and eight weeks respectively for the first and second experimental periods.

Variations in calcium, sodium, potassium, manganese and chloride were trivial. No meaningful correlation was seen between viable cell counts and soluble ions for iron (up to 3.4 ppm) or for phosphate (up to 0.1 ppm). Since soluble iron is subject to precipitation through oxidation to ferric species and through precipitation as iron (II) sulfides, clear correlations with soluble iron would have been surprising. Phosphate is an essential nutrient for microbial growth but is very efficiently collected and concentrated by biomass. It may be that phosphates collected by the biofilm community are retained through recycling lessening the dependence of the community on soluble phosphates from the water stream.

Neither variations in total organic carbon (up to 40 ppm), variations in total suspended solids (up to 2400 ppm) or overall alkalinity influenced the sessile population.

An excursion in the nitrate concentration during the first test period corresponded to an apparent suppression in the viable counts for the sessile sulfate reducing bacteria, Figure 1. While the correlation is weak given the scatter in the data, nitrate is known to suppress sulfate reducing activity (3,4).

An excursion in the soluble sulfate concentrations appeared to promote both sulfate reducers which depend on this ion for their metabolism and the associated population of acid producing bacteria, Figures 2 and 3. These latter organisms are heterotrophs able to use more complex carbon nutrients to generate organic acids. Convenient assays have been developed for these organisms ("APB") through research programs looking at pipeline corrosion elsewhere (5). In this situation, the "APB" assay has been used simply as a second index of microbial growth and reflects in part the population of other organisms associated with the biofilm. Given the limited sulfate normally present in these barium rich brines, it is possible that the sulfate reducing bacteria present are sulfate limited and respond readily to increases in soluble sulfate.

A surprising inverse correlation was noted for magnesium ion concentration and viable counts for sulfate reducing bacteria, Figure 4. If real, this correlation has no ready explanation at this time, although attention of the ion balance across microbial membranes may be implicated.

A correlation might have been expected between the number of sulfate reducing bacteria present and soluble sulfides. In effect this was noted in only one of the experimental periods, Figure 5. Given the possible precipitation of sulfide with various metal ions present at higher concentrations, it is possible that the relationship evident at low sulfide levels is being obscured by precipitation phenomena above 3 ppm sulfide. Alternately, low sulfides may arise where low sulfate has limited the sulfate-reducing population. Where sulfate is in excess and other factors are limiting, sulfide

production need no longer correlate with bacterial cell counts.

Adaptation of Microbial Populations

SRB and APB assays can be used as crude tools to characterize a mixed population since the numbers obtained represent substantially different groups of organisms. In terms of these assays the biofilm community at the field test facility has remained remarkably constant over a three year period, Figure 6, with the sulfate reducing bacteria dominating acid producing bacteria in the community.

Bull plugs were colonized at ten different locations in adjacent field units at both waterplants and free water knock outs. While these other units are operated independently, the fluids and conditions are similar to those found at the test facility. Colonized bull plugs were removed and transported to the field test facility in sealed containers filled with their native brine. The plugs were then inserted into the test facility for the second test period of eight weeks.

In nine of the ten cases, the mixed communities assumed the SRB/APB ratio typical of the field test unit population. The example shown in Figure 7 is representative. The sulfate reducing bacteria in the unit 5 free water knock out population increased dramatically on exposure to water and conditions found in the field test facility while a population from unit 5 water plant decreased in acid producing bacteria to achieve the same final distribution. A population from unit 14 free water knock out responded by increasing in sulfate reducers and decreasing in acid producers to achieve a similar final distribution, Figure 8. The only anomalous case was seen for the waterplant population from unit 14 waterplant which shifted to an APB rich mixed population in the field test facility, Figure 8.

Whether these shifts represent a simple change in the ratio of organisms originally present on the bull plugs or the overrun of that community by organisms native to the field test community is not discernible by the simple assays used here. More sophisticated tools are needed to assay the fate of specific organisms in either the introduced or native populations.

Overall the implication is that the water chemistry and physical conditions found in the water piping foster mixed sessile communities of definite composition and that communities introduced from similar environments elsewhere will shift in a matter of weeks to a favoured composition presumably optimized for that site. The anomalous case seen for unit 14 waterplant suggests that the favoured composition is however not unique and may be upset by the introduction of organisms from elsewhere. Shifts seen in these experiments are far more dramatic than those induced by modest variations in water chemistry (Figures 2 to 4) above.

IRON SULFIDES AND THE CORROSION MECHANISM

Iron sulfides are a complex group of materials which play a key role in microbial corrosion sites (6). Electron microscopy using both SEM and TEM supplemented by selected area electron diffraction (SAED) and energy dispersive X-ray analysis (EDX) was used to examine the biofilm. These results will be described in detail elsewhere but a summary of the findings is presented here.

Scanning electron micrographs revealed two sorts of surface deposits on the internal steel surfaces in the test spools. A thin corrosion deposit free of bacteria covered most of the coupon area with thicker deposits of cake like appearance randomly distributed on the surface. These thicker deposits were generally relatively smooth with distinct areas appearing to be more porous. Bacteria were commonly seen in these porous domains.

Thin sections examined by transmission electron microscopy showed a variety of organisms were embedded in the corrosion deposit matrix with the highest concentration of organisms being seen near the top surface of the deposits. Intact cells were found deeper in the matrix along with heavily mineralized ghost cells.

The bulk of the corrosion product consisted of amorphous chloride rich iron sulfide (EDX result) with about 10% of the material appearing to be poorly ordered mackinawite ($FeS_{0.9}$) (SAED result). Chloride is apparently retained in the matrix to charge balance the non-stoichiometric ratio of sulfide to iron in the sulfide deficient precipitate.

The presence of viable sulfate reducing bacteria in an iron sulfide rich matrix on the steel surface provides the elements of a severe corrosion mechanism in which the iron sulfide forms a galvanic couple with the steel surface sustained and extended by the sulfate reducing bacteria (6). In this scenario, the sulfate reducers remove electrons from the high surface area iron sulfide matrix and use this reducing power to reduce sulfate to sulfide, a reaction couple which yields metabolic energy to these organisms. The electrons arise of course through the corrosion of steel (Fe(0) going to Fe (II) + 2 electrons). The liberated ferrous iron precipitates with the released sulfide to generate more iron sulfide and so extend the corrosion cell. This is a refinement of the cathodic depolarization mechanism first proposed by Von Wolzogen Kuhr and van der Vlugt in 1934 (7) for external corrosion of pipelines in black, wet soils rich in sulfate reducing bacteria.

Other microbially influenced corrosion mechanisms may or may not be operating in this system but the above mechanism has been shown in the lab to account for severe corrosion with pitting penetration rates up to 0.7 mm/year (6).

CONCLUSIONS

Mixed sessile microbial populations are intimately associated with corrosion deposits in oilfield water handling systems. In the high brine environment of the Wainwright field waterplants, sulfate reducing bacteria predominate and lead to a biofilm and corrosion products rich in iron sulfide and chloride. The composition of the population at least in terms of the ratio of acid producing bacteria and sulfate reducing bacteria is relatively constant over time and optimized according to the water chemistry and conditions present. These populations are self sustaining, resist biocides and are mildly sensitive to local variations in water chemistry especially with respect to sulfate, nitrate and surprisingly magnesium. Such stable systems are bound to cause corrosion through a known and documented cathodic depolarization mechanism in which an iron sulfide/steel galvanic couple is enhanced and extended by bacterial action.

ACKNOWLEDGEMENTS

We are grateful to our partners and collaborators; Ed Rogoz at Husky Oil Operations Limited, Dr. Ron McCready formerly at Canmet, EMR and the staff under Professor Terry Beveridge at the regional electron microscopy facility at the University of Guelph as well as our many colleagues at NHRC for their assistance and support.

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Table 1: Injection water chemistry at start of the first and second experimental runs		
	WATER CONSTITUENTS (ppm)	
PARAMETER	**FIRST EXPERIMENT Run #1**	**SECOND EXPERIMENT Run #2**
CATIONS		
Na^+	25000	24000
K^+	510	320
Ca^{2+}	2200	2000
Mg^{2+}	1000	1000
Fe^{2+}	0.24	0.37
Mn^{2+}	0.50	0.28
ANIONS		
Cl^-	53000	39000
SO_4^{2-}	0.10	0.10
NO_3^{1-}	0.10	0.10
PO_4^{3-}	0.03	0.20
S^{2-}	1.10	0.50
WATER QUALITY		
Alkalinity (as $CaCO_3$)	510	450
Total Suspended Solids	2400	210
Total Organic Matter	20	14

FIGURE 1: Scatter plot of sulfate-reducing bacterial counts and nitrate concentrations in the first experiment

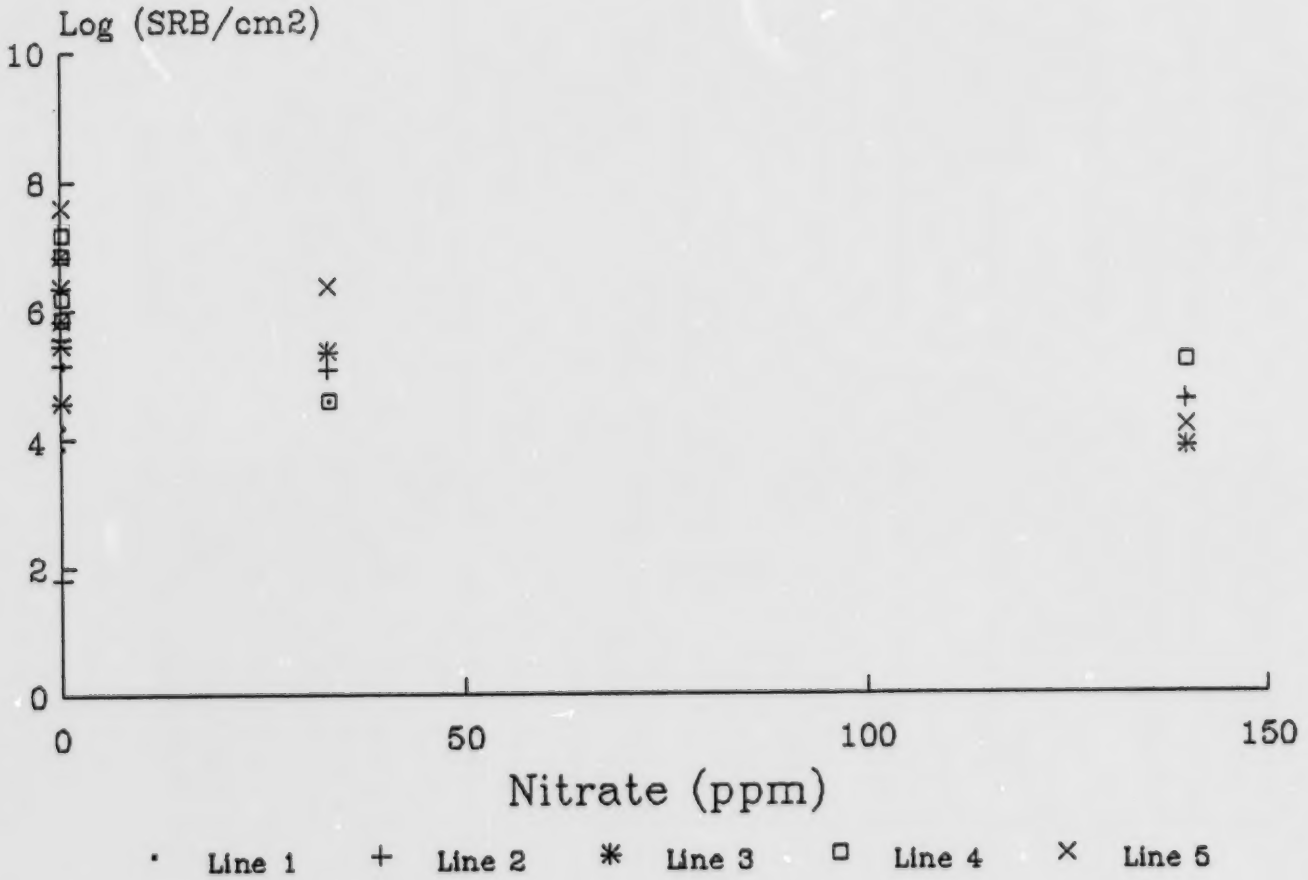


FIGURE 2: Scatter plot of sulfate-reducing bacterial counts and sulfate concentration in the first experiment

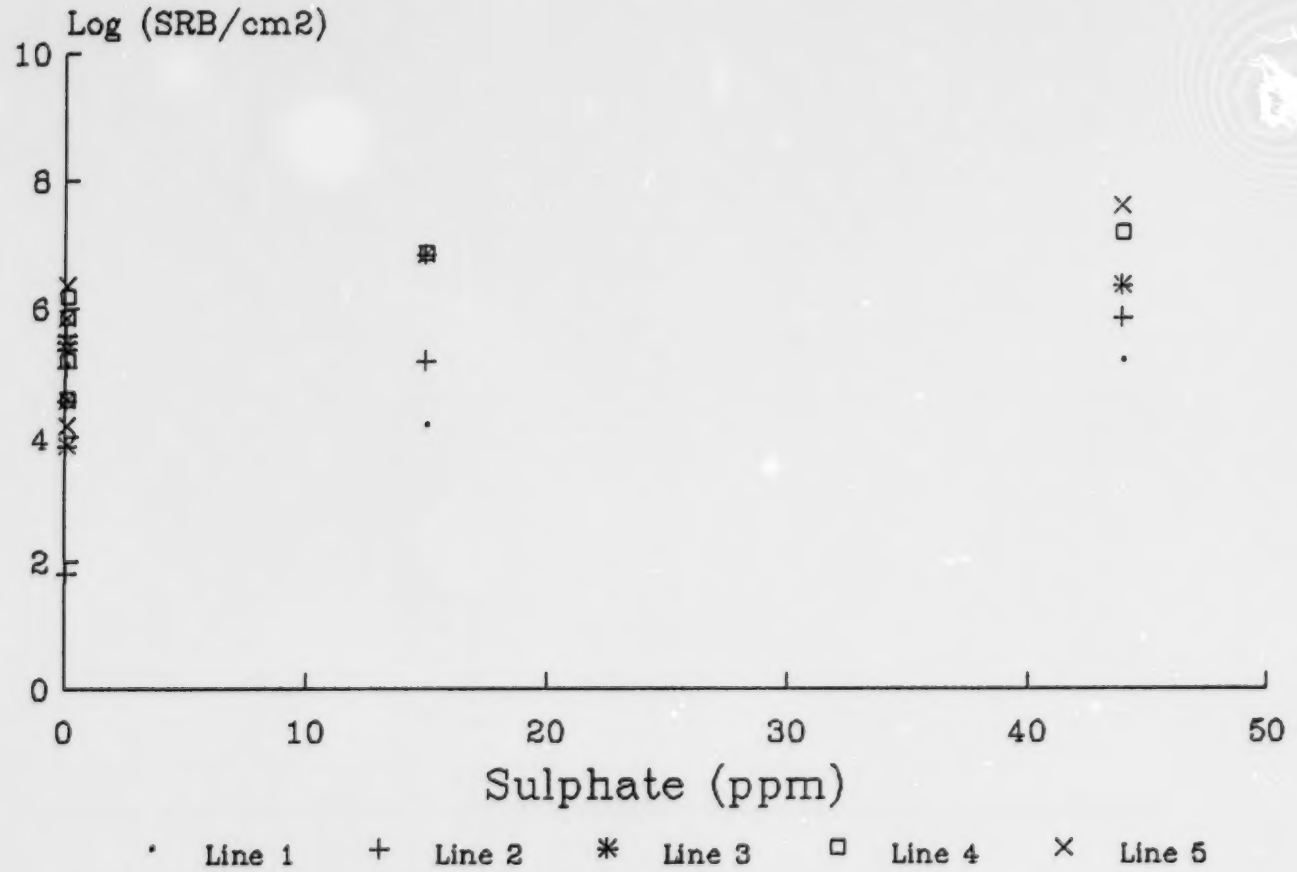


FIGURE 3: Scatter plot of acid-producing bacterial counts and sulfate concentrations in the first experiment

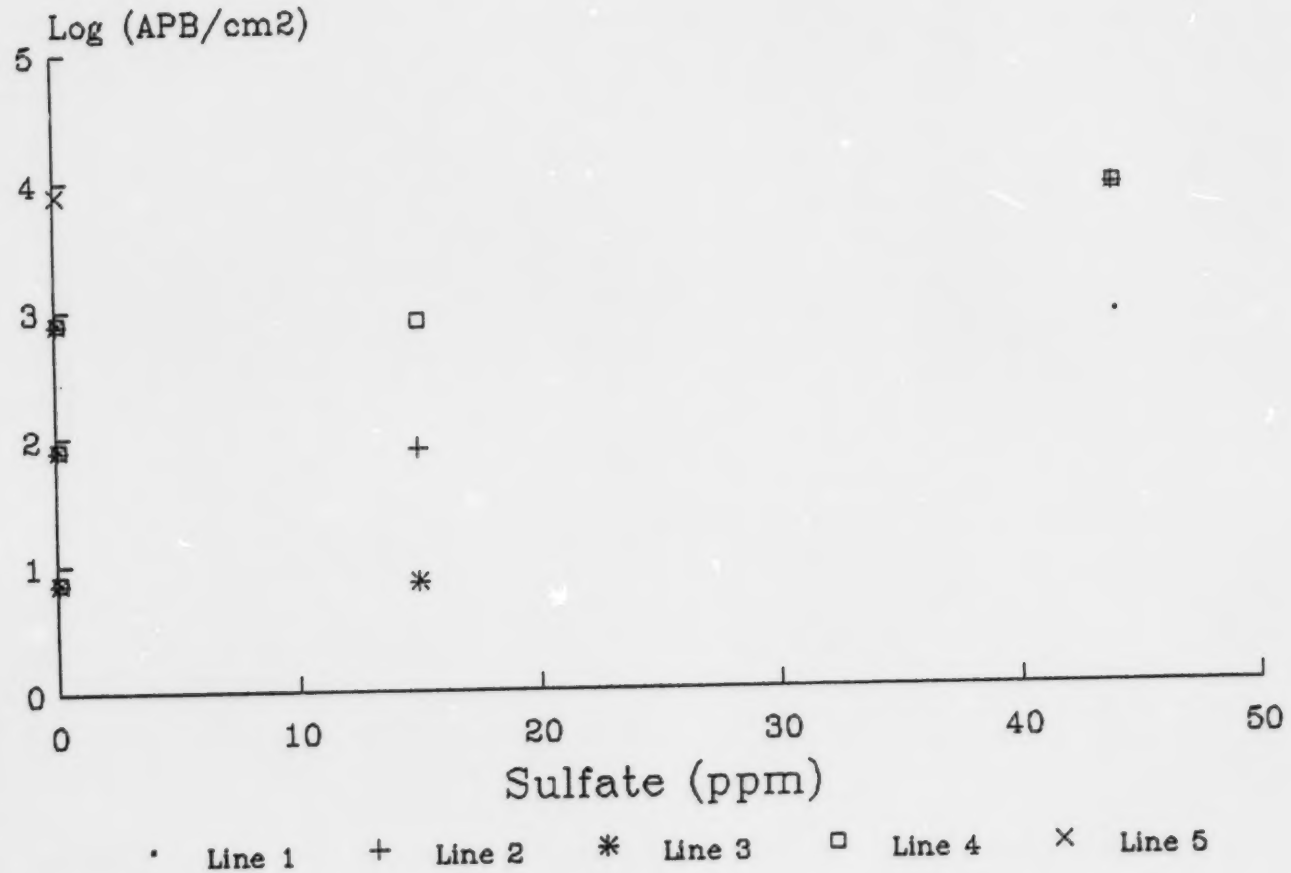


FIGURE 4: Scatter plot of sulfate-reducing bacterial counts and magnesium concentrations in the first experiment

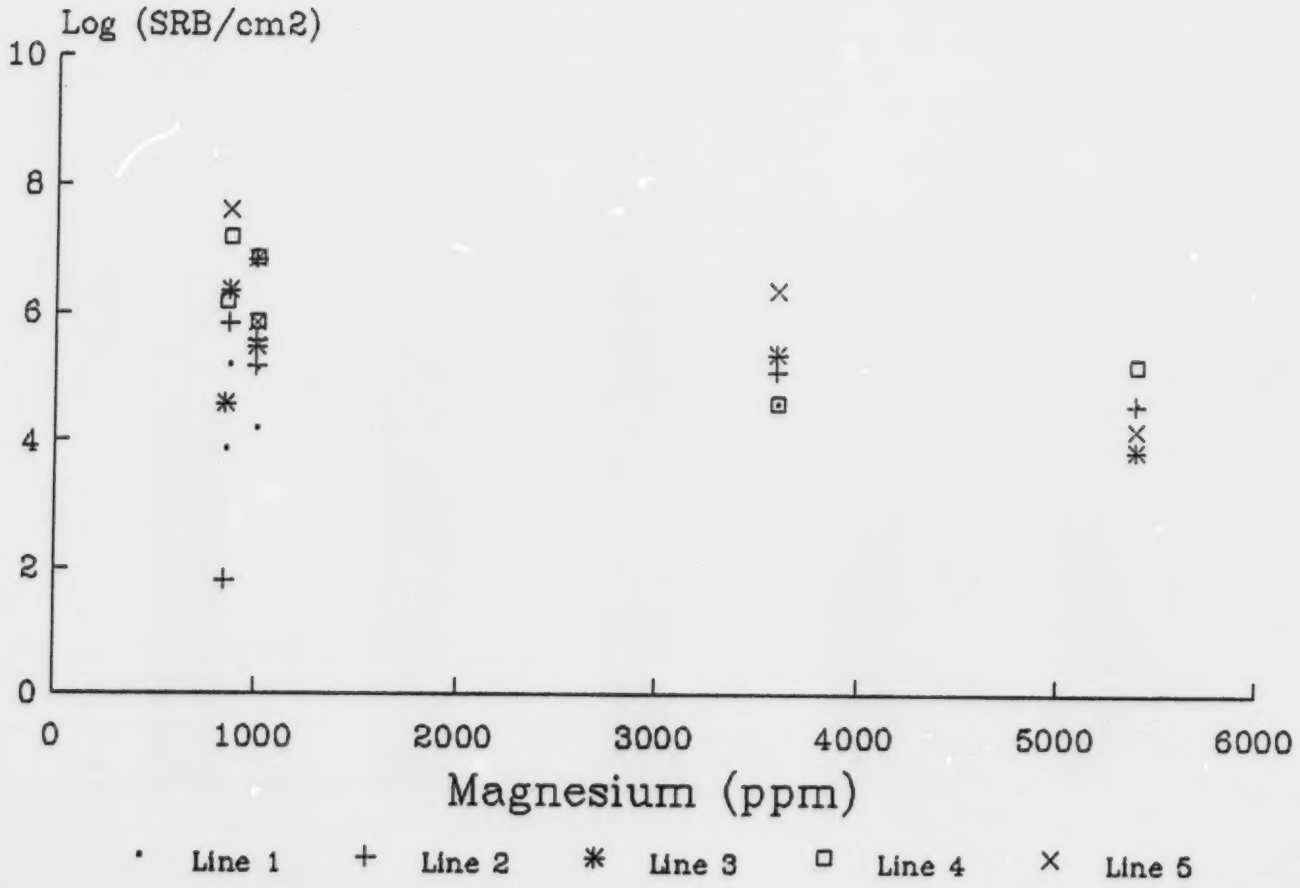


FIGURE 5: Scatter plot of sulfate-reducing bacterial counts and sulfide concentration

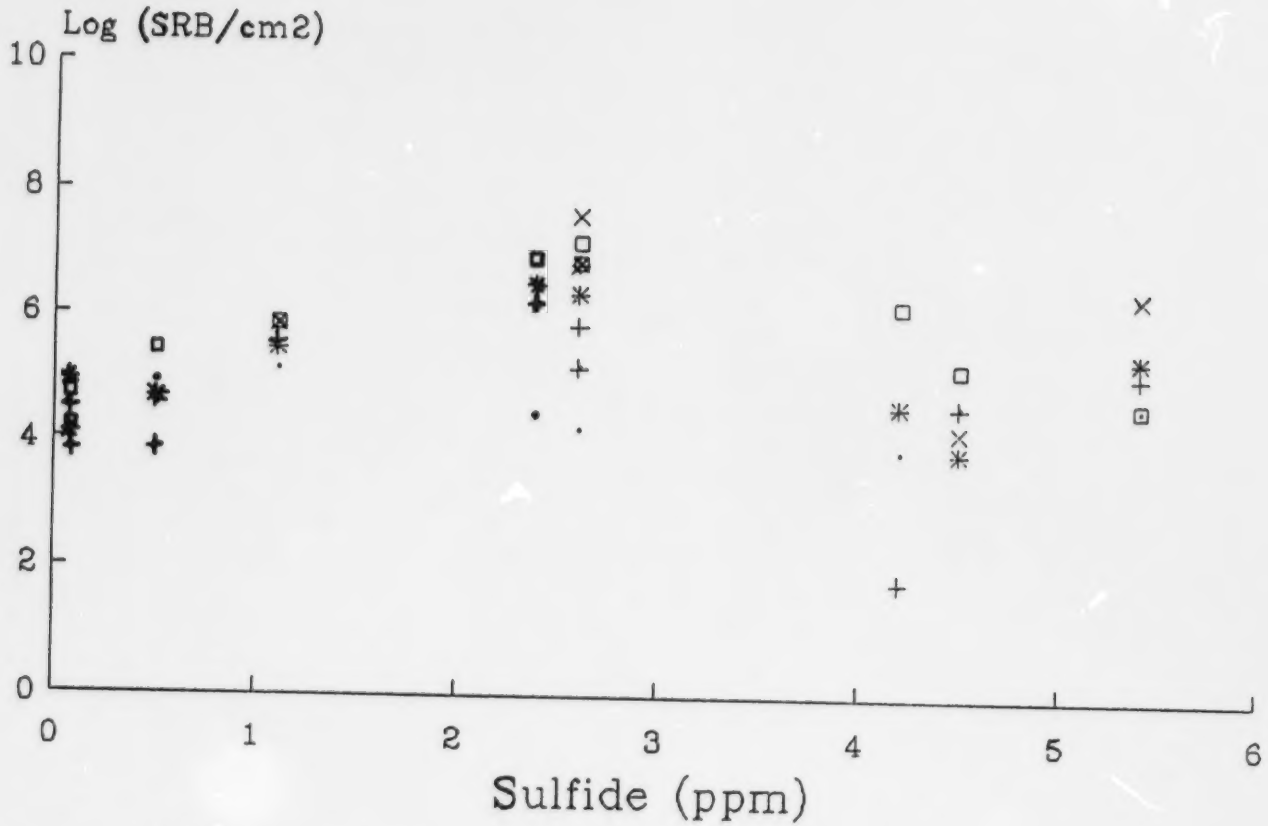


FIGURE 6: Mean values of viable counts for sulfate-reducing and acid-producing bacteria after four weeks of colonization at the field test facility

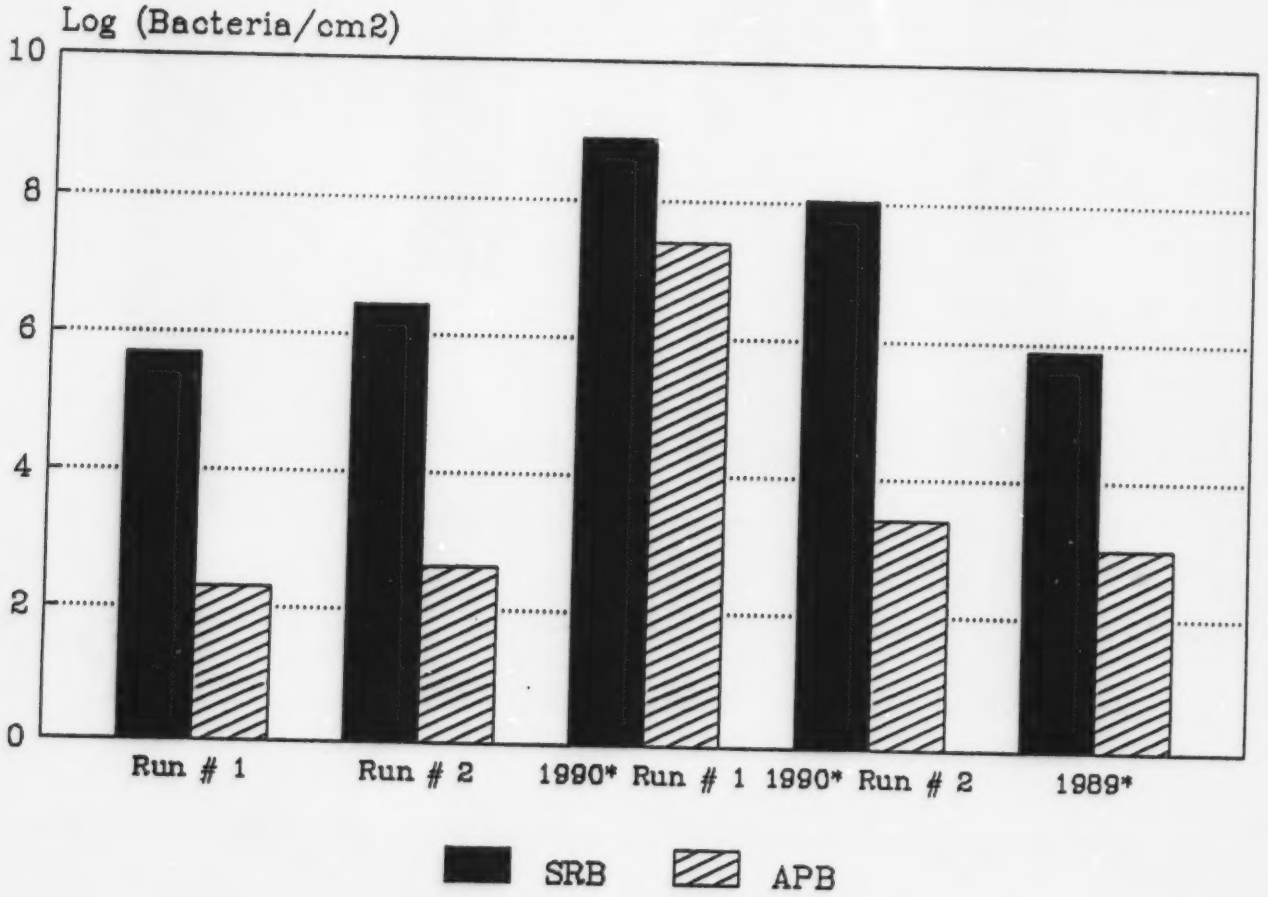


FIGURE 7: Bacterial counts on bull plugs from Unit #5 before and after exposure at the field test facility

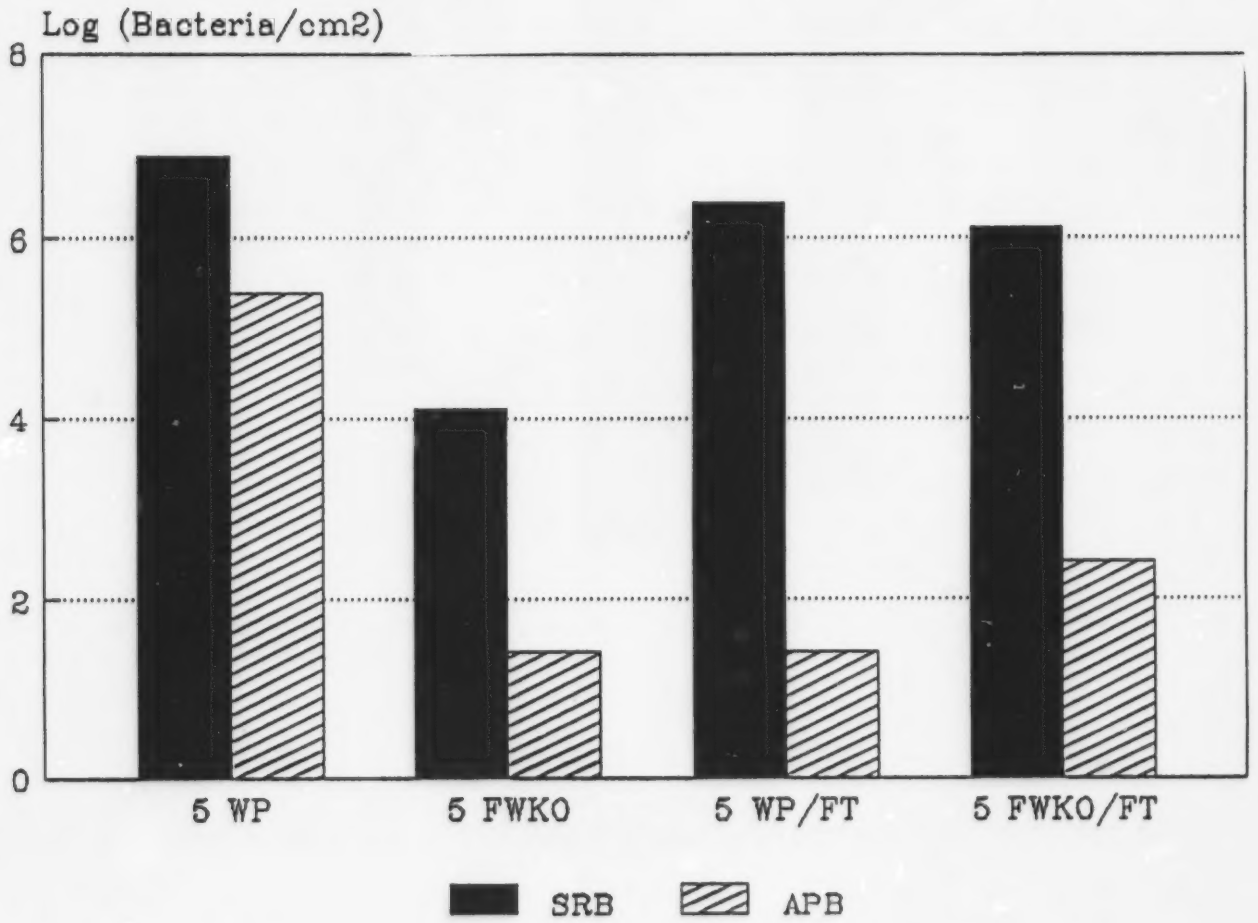
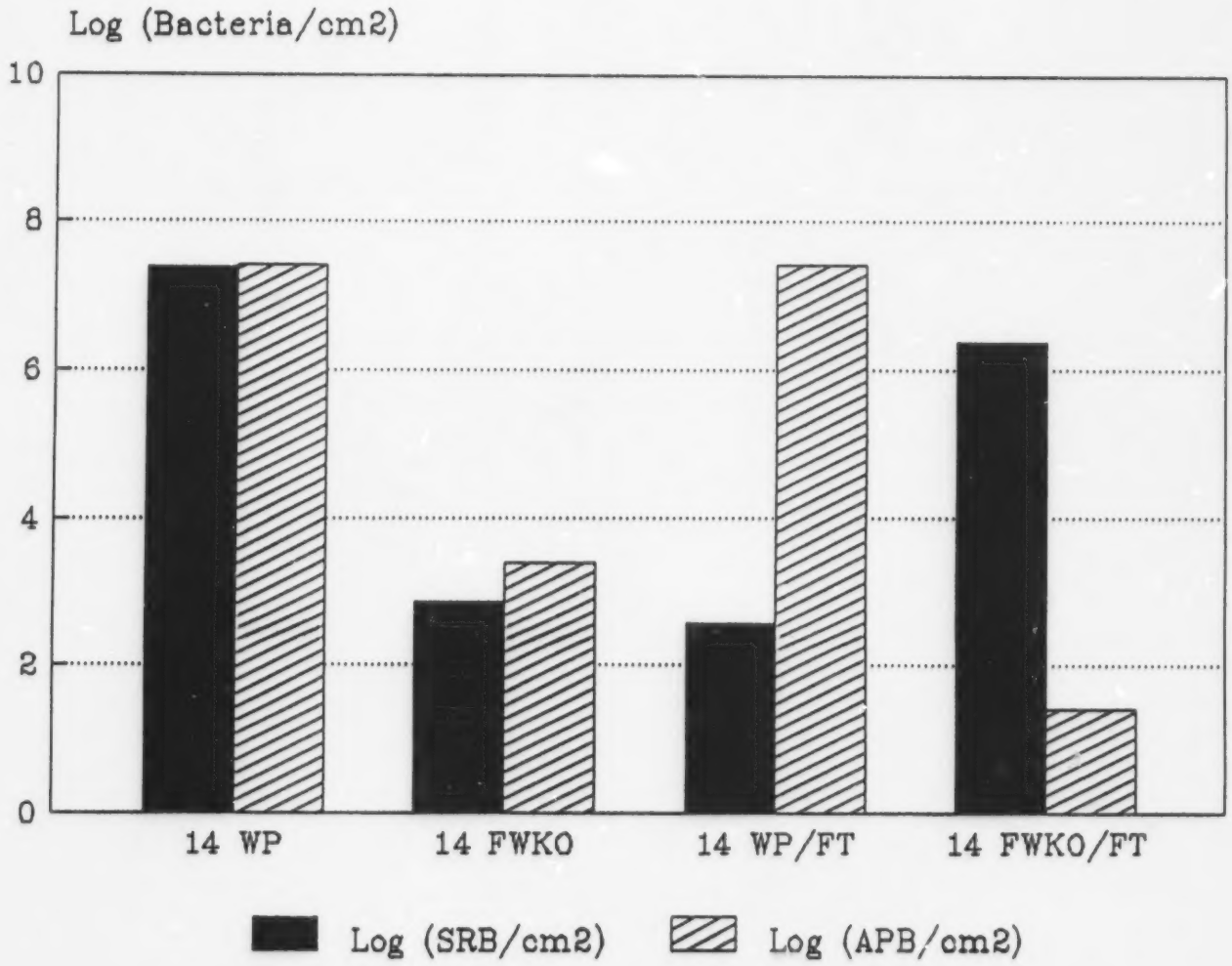


FIGURE 8: Bacterial counts on bull plugs from Unit #14 before and after exposure at the field test facility



MICROBIALLY INFLUENCED CORROSION ON A TEST PIPE

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ABSTRACT

The influence of bacteria in the development of pipeline corrosion, under disbonded coatings has been investigated in a laboratory study for 12 months. The 508 mm (20 in.) diameter test pipe was pressurized by a simulated one-year gas pipeline spectrum. The pipe possessed natural and artificial stress corrosion cracks. Environmental cells containing Alberta or Ontario soils were placed around the pipe. Growth of the artificial cracks was followed by a direct current potential drop technique. The enumeration of different microbial metabolic groups and measurements of metabolic activity, together with analyses for organic acids and ions were performed on aqueous samples recovered from under disbonded coatings. Furthermore, the water content and redox potential of the surrounding soils were periodically monitored. The influence of bacteria, together with chemical and physical parameters, on the growth of artificial stress corrosion cracks on a pipeline steel will be discussed.

CORROSION D'UNE CONDUITE D'ESSAI LIÉE A L'ACTIVITÉ MICROBIENNE

RÉSUMÉ

L'influence des bactéries sur la corrosion des pipelines, sous des revêtements détachés, a été étudiée en laboratoire pendant un intervalle de douze mois. La conduite d'essai d'un diamètre de 508 mm (20 po.) a été pressurisée par une séquence de pression simulant l'utilisation d'un gazoduc pendant une période d'un an. La conduite présentait des fissures naturelles et artificielles de corrosion sous contrainte. Des cellules environnementales renfermant des sols albertains et ontariens ont été placées autour de la conduite. La croissance des fissures artificielles a été suivie par une méthode de chute de potentiel en courant continu. On a énuméré des groupes de micro-organismes présentant différents métabolismes en plus d'effectuer des mesures de l'activité métabolique ainsi que des dosages des acides organiques et des ions dans des échantillons aqueux recueillis sous des revêtements détachés. De plus, la teneur en eau et le potentiel d'oxydo-réduction des sols avoisinants ont été périodiquement surveillés. L'influence des bactéries et des paramètres chimiques et physiques sur la croissance des fissures artificielles de corrosion sous contrainte dans un acier pour pipeline sera discutée.

INTRODUCTION

The influence of microorganisms on the corrosion of iron or steel objects buried in the ground has been described as early as 1910 (Gaines, 1910). Since that time there have been numerous reviews which have discussed the influence of microorganisms on the corrosion of metals (Costello,1969; Hamilton, 1985; Iverson,1987; Miller and King, 1975). Despite this attention, mechanisms describing microbially influenced corrosion are still not fully established. The ability of microorganisms to produce metal loss and pitting corrosion is widely acknowledged (Booth and Wormwell,1962; Postgate, 1984) . In contrast the role of bacteria in stress corrosion cracking (SCC) of high strength steels is not well understood. SCC is the cracking of a metal due to the combined action of high stress and corrosion (Dix, 1940). Basically, stress corrosion cracks grow as a result of localized anodic dissolution, or hydrogen embrittlement at the crack tip, or a combination of both mechanisms. Studies directly implicating microorganisms in SCC are few in number. A laboratory study has indicated that the hydrogen and organic acid producing Clostridium acetobutylicum will induce the cracking of a high strength steel wire (Ford and Mitchell, 1991). Indirect evidence is provided in a review by Cragnolino and MacDonald (1982) in which the cracking of stainless steels in the presence of a number of sulphur species including hydrogen sulphide, which is produced by the sulphate-reducing bacteria, was reported. Results of a field study suggested that sulphate-reducing bacteria may have been involved in the SCC of pipe in a gathering system (Kiefner and Eiber, 1987). The aim of the present laboratory study is to monitor the growth of natural and artificial cracks in a laboratory pipeline model, and to correlate any crack growth with microbial community structure and activity.

MATERIALS AND METHODS

Test Pipe

A full-size pipe section, excavated by a Canadian company after seventeen years of service was used as the test pipe. The test pipe which was composed of X65 (Grade 448) steel, was 4.5 m long, with an outer diameter of 508 mm (20 in.) and a 6.4 mm thick wall. The test pipe was subjected to a pressure sequence identical to that of a selected gas pipeline. Three double chambers were placed around the test pipe (Figure 1). Each chamber consisted of two cells. Each cell contained approximately 0.1 m^3 of soil obtained from pipeline excavation sites. Odd number cells contained an Ontario soil while even number cells contained an Alberta soil. Different conditions were applied to each double chamber. Cells 1 and 2 were operated under free corrosion conditions (initial values -700 to-750 mV versus a saturated calomel electrode). The pipe section in Cells 3 and 4 was placed under cathodic protection (initial values -800 to -950 mV versus a saturated calomel

electrode). The pipe section in Cells 5 and 6 was also placed under cathodic protection. In addition, Cells 5 and 6 contained an iron sulphide layer approximately 4 inches below the soil surface. X-ray diffraction analysis indicated that this layer contained sulphur, pyrite (FeS_2) and greigite (Fe_3S_4). This added layer also contained straw and a microbial community including sulphate-reducing bacteria. The source of this heterogenous layer is a laboratory model which is described elsewhere (Béchard et al., in press). The soils have been maintained under water saturated conditions. Sets of artificial cracks are located on the pipe exterior at the 3,6,9 and 12 o'clock positions of each chamber. Each set consisted of two nearly semi-elliptical cracks which were cut to a depth of 1.5 and 2.3 mm. At each position, two sets of cracks were located. One set is under a simulated disbonded coating while the second is freely exposed to the soil environment. The electrochemical potential on the pipe surface was measured with the aid of Luggin capillaries, which were placed between the cracks. The depth growth of these cracks was assessed by the Direct Current Potential Drop (DCPD) technique, using a 128-channel system (MDT Engineering, Waterloo, ON, Canada). This technique relies on each crack being straddled by two pairs of wire probes. The introduction of direct current through the outside pair of probes will create a potential field across the crack. Crack growth will change the potential field and this will be monitored by the inside pair of wire probes.

To avoid disturbing the solution over the cracks, a dummy disbonded tape was placed next to the crack locations for electrolyte extraction. Trapped water samples were removed from these locations for chemical and microbiological analysis. The sampling has been divided into three time periods. Period 1 extended from Sept. 1990 - Jan. 1991, Period 2 from Feb. - July 1991 and Period 3 extends from Aug. - Jan. 1992. A total of 24 trapped water sites were located on the test pipe. Each cell also contained a colony of natural stress corrosion cracks. The number of cracks and the crack length distributions were measured at the start of the test, and will be measured again at its completion using image analysis. The test began September 12, 1990 and will terminate January 12, 1992. It is being conducted at ambient temperature (20-25 °C).

Water Content and Redox Potential

Time domain reflectometry (Topp et al., 1984) was used to obtain measures of the soil water content at depths of 10.2 cm (4 in), 17.8 (7), 25.4 (10), 41.9 (16.5) and 77.5 cm (30.5 in) in each chamber. Redox potential (Eh) measurements were conducted at the same depths using glassy carbon electrodes and a saturated calomel reference electrode.

Bacterial Enumeration and Activity

A 1.5 ml liquid sample from each dummy disbondment site was used to inoculate one type of medium. Sample collection was repeated at an interval of not less than three days. Ten-fold

dilutions with five tubes per dilution were used to calculate the most probable numbers in each of the following media: total aerobic viable bacteria were enumerated with medium which contained (per litre of double deionized water) 1.6 g of nutrient broth (Difco, Detroit, Mich.), and 20.0 g of agar (Difco, Detroit, Mich.), pH 7.2. Dilution tubes contained 0.1% pyrophosphate, pH 7.2. Incubation was conducted at 28 °C for 72 hours. Acid producing bacteria were enumerated with medium of the following composition(per litre of double deionized water): 5.0 g of dextrose (Difco, Detroit, Mich.), and 16.0 g of phenol red broth (Difco, Detroit, Mich.), pH 7.2. Colour change was noted after 24 hours incubation at 28 °C. A modification of Postgate medium B (Postgate, 1984) which contained (per litre of double deionized water): 0.5 g of KH_2PO_4, 1.0 g of NH_4Cl, 1.0 g of $CaSO_4$, 2.0 g of $MgSO_4.7H_2O$, 1.75 g of sodium lactate, 1.28 g of sodium acetate, 1.0 g of yeast extract, 1.0 ml of 0.1 % resazurin, 10.0 ml of 2.5% $Na_2S.9H_2O$, pH 7.2, was used to enumerate sulphate reducing bacteria. Blacking of the medium was recorded after 4 weeks incubation at 30 °C. Denitrifying bacteria were enumerated with the medium of Alexander (Alexander, 1965). Incubation was conducted for seven days at 30 °C.

Hydrogenase activity was measured with the Caproco hydrogenase test (Caproco (1987) Ltd., Edmonton, AB, Canada). Enzymatic activity was graded by a scale in which values of 1-3 represented 0 to 0.05 nanomoles H_2 uptake per minute, and 4-6 corresponded to 0.5 to 5 nanomoles H_2 uptake per minute.

Analytical Procedures

Liquid disbondment samples were collected and filtered through 0.2 polycarbonate filters (Nuclepore Canada Inc., Toronto, ON, Canada), and stored in polyethylene vials at -2 °C. Organic acids were monitored with a SP8800 liquid chromatograph (Spectra-Physics, San Jose, Calif.) The 222ORH-801 organic acids column (Interaction Chemicals Inc., Mountain View, Calif.) was maintained at 40 °C, and the solvent (0.01 N H_2SO_4) had a flow rate of 0.8 ml/min. Detection by direct UV absorption (Spectra-Physics Spectra 200) occurred at 215 °C. Organic acid values represent the summation of organic acids and their conjugate bases. Anions were determined by ion exclusion chromatography using a permanently coated anion exchange column (Barkley et al. 1987). Metal cations, except sodium and potassium were measured using by a JY38 inductively coupled plasma-emission spectrometer (Jobin Yvon, France). Sodium and potassium were determined by an IL551 atomic adsorption spectrophotometer (Instrumentation Laboratory Inc., Wilmington, Mass.).

RESULTS AND DISCUSSION

Field studies of stress corrosion cracking are very limited. In this laboratory study a number of physical parmeters which are known to be conducive for general and pitting corrosion, were monitored in order to relate them to stress crack growth. The

assessment of microbial and related chemical and physical parameters has been divided into three periods. Period 1 extended from Sept. 1990 - Jan. 1991, Period 2 from Feb. - July 1991 and Period 3 will terminate Jan. 1992. The pH values of the trapped water at the disbondment sites have been routinely measured at approximately three week intervals. In the cells under free corrosion (1,2) pH values were 7.0 ± 0.5. In cells with cathodic protection (3-6) the pH values were 7.5 ± 0.5. On average the pH of the trapped water in cells containing the Alberta soil appeared to be slightly higher than in cells containing the Ontario soil. No consistent variation in pH with respect to the position on the pipe has been observed.Throughout the experiment the layers which surround the test pipe have been saturated. Water content values were 48 ± 3% for the Ontario soil and 72 ± 6% for the Alberta soil. Booth et al. (1967) indicated that soils with a water content above 20% and a redox-potential at pH 7, below 400-430 mV, would be considered to be environments conducive for the general pitting corrosion of iron and steel. All cells meet the water content requirement, and considering the surrounding soil Eh values (Figure 2), all sites of cell 1 (10, 16.5, and 30.5 inch depths), the 3 and 9 o'clock positions (16.5 inch depth) of cell 2, the 12 and 6 o'clock positions of cell 4 (10 and 30.5 inch depths) , and the 3,6 and 9 o'clock positions (16.5 and 30.5 inch depths) of cell 6 appear to possess values within the range suitable for pitting corrosion. It appears that these physical parameters may exert the same influence on stress corrosion cracking. DCPD measurements have recently suggested that crack growth is occurring at the 3 o'clock position of Cell 2.

The role of sulphate-reducing bacteria (Iverson,1987), acid producing bacteria (Pope et al., 1989), and denitrifying bacteria (Abdrashitova et al., 1988) in the corrosion of steels has been reported. Therefore these groups are being monitored in order to see if their presence correlates with stress corrosion crack growth. Aerobic heterotrophic bacteria are being enumerated in order to provide an indication of total bacterial numbers. The findings are presented graphically in Figures 3 and 4. Blank pie sections represent the absence of data. The aerobic heterotrophic bacteria were the predominant group at (13 of 18) 72 per cent of the sites (with complete data) in Period 1 but only (13 of 24) 54 per cent of the sites during Period 2. In Period 1 there was no apparent distribution of aerobic heterotrophic bacteria around the pipe surface. However, in Period 2, with the exception of Cell 5, this group was always highest in numbers at the top of the pipe. This trend concurs with the Eh readings, which generally indicate that the cells became more anaerobic with respect to soil depth and time. During Period 1 the denitrifying bacteria predominated at only one trapped water site (Cell 5, 6 o'clock). However by Period 2 this group predominated at over one third of the sites. Similar to the aerobic heterotrophic bacteria total numbers of denitrifying bacteria were highest at the top of the pipe by Period 2. In addition the total number of denitrifying bacteria had increased by more than a factor of five, at 71 percent of the sites (with complete data), when Period 2 is compared to Period 1. Occasionally

the sulphate-reducing bacteria were the predominant group. It is interesting to note that when the sulphate-reducing bacteria predominated during Period 1 (Cells 2,4,6), it was in the Alberta soil, while during Period 2 it was in Cell 3 which contained the Ontario soil (Figures 2 and 3). Relative to Period 1, the sulphate-reducing bacteria generally increased their total numbers in Period 2 (Table 1 and 2). The data of Period 2 also indicate that in 5 of the 6 cells, sulphate-reducing bacteria numbers where higher at the top and bottom of the pipe in comparison to the disbondment sites on the sides of the pipe (3 and 9 o'clock). The data of Period 2 also demonstrate that average sulphate-reducing bacteria numbers were eight times higher in the Ontario soil compared to the Alberta soil. The acid producing bacteria were never predominant in either period. Their low numbers relative to the other bacterial groups prevented their appearance on the pie charts except for 3 sites in each of Periods 1 and 2. During Period 1 the numbers of acid producing bacteria were always exceeded by the sulphate-reducing bacteria. In Period 2, with the exception of two sites (12 o'clock position of Cells 4 and 6), the numbers of sulphate-reducing bacteria had considerably increased relative to the acid producing bacteria. However it is also noteworthy that in Period 2, for the first time, there were two sites (12 o'clock position of Cells 4 and 6) at which the acid producing bacteria exceed the sulphate-reducing bacteria. By Period 2 it was obvious that the acid producing bacteria were highest at the top of the pipe, and tended to decrease drastically at the lower depths. This distribution profile was not apparent in the Period 1 data.

The concentration of formic, acetic, pyruvic, propionic, butyric, and lactic acids were determined in Period 2. A summation of these organic acids at each disbondment site is presented in Figure 5. Acetic acid and formic acids were the predominant acid at an equal number of sites. Butyric acid was not detected at any of the sites. There appeared to be some tendency for the organic acids to be at the highest concentration at the top position of the pipe. This would be in agreement with the enumeration data which indicated that during Period 2 the acid producing populations were generally the largest at the 12 o'clock position of the pipe. Compared to a report of acetic acid concentrations of 50-65 mM in certain separators and storage tanks suffering from non-SCC corrosion (Dr.D. Pope, personal communication) the cumulative acid concentrations at the disbondment sites in the SCC model were relatively low, ranging from 0.29-2.94 mM. However, it is possible that low levels of acid observed in our experiment may even be conducive to the development of stress corrosion cracking. Higher levels would produce general or pitting corrosion, but low levels may be sufficient to sustain stress corrosion crack growth.

The concentrations of specific ions in the trapped water of Period 2 have been averaged for each cell (Figure 6). Total iron was not detected and therefore was less than 0.0022 meq/L, potassium was less than 0.026 meq/L, manganese was less than 0.0022 meq/L, nitrite was less than 0.022 meq/L, nitrate was less than 0.016 meq/L, phosphate was less than 0.032 meq/L, and thiosulphate

was less than 0.018 meq/L at all sites. Cell 6 possessed higher average values of chloride, sulphate, calcium, magnesium, and sodium ions than all other cells. On average, sulphate levels were four times higher in the Alberta soil compared to the Ontario soil. The low levels of sulphate in Cells 1 and 5, in comparison to sister Cell 3, suggest that microbial utilization of the sulphate may have occurred in these two cells. With the possible exception of Cell 6 the concentration of the cations, approached values observed in the trapped water of SCC sites in the field (unpublished results of a major pipeline company). However the concentration of chloride and especially sulphate (1.39-36.28 meq/L) exceeded the field values in all cells. In the field, sulphate concentrations ranging from 5 to over 25 meq/L were only observed in the groundwater in the region of the pipeline, whereas in the trapped water at SCC sites, the sulphate concentration ranged from less than 0.1 to 0.50 meq/L. This suggests that the high levels observed in the trapped water in our test system indicate a level of immaturity which bacterial activity may rectify with time.

The utilization of hydrogen by hydrogenase containing bacteria has been considered to enhance metal corrosion via cathodic depolarization. Hydrogenase containing organisms have been reported to produce serious corrosion (Pankhania, 1988). During Period 1 hydrogenase activity was detected in all cells, but not at all locations in each cell. The Ontario soil (Cells 1,3,5) consistently showed activity at the bottom of the pipe (6 o'clock position) while the Alberta soil (Cells 2,4,6) consistently showed activity at the top of the pipe (12 o'clock position). In addition the Alberta soil demonstrated activity at the 9 o'clock position in Cells 8 and 10. The pipe in these cells was cathodically protected and the 9 o'clock position was the location of the sacrificial anode. Cell 2 which did not have a sacrificial anode at the 9 o'clock position did not demonstrate any hydrogenase activity.

CONCLUSIONS

1. The microbial data indicate that a large and rich community of microorganisms are present under the disbonded coatings in our laboratory study. This is consistent with the observation of mixed bacterial communities under disbonded tape at field corrosion (non-SCC) sites (Worthingham *et al.*, 1986). Thus, our laboratory pipeline model appears to simulate the field environment.

2. A number of trends are evident in the chemical, physical and microbial data, and the direct current potential drop technique measurements are encouraging at several sites. In particular, DCPD measurements at the 3 o'clock position of Cell 2 are indicating crack growth of approximately 5×10^{-8} mm/s. The dummy disbondment located next to the cracks at this position is not dominated by sulphate-reducing or acid producing populations, but these metabolic groups are present.

The absence of cathodic protection in this cell suggests that its absence may be necessary to permit crack growth. In this respect our results may mirror observations in the field, in which SCC was observed to occur under disbonded coatings where the pipeline surface was shielded from cathodic protection. Regardless of these initial findings, the fractographic analysis using optical and electron microscopy that will be undertaken at the completion of the test on January 12, 1992, is required before any unequivocal statements can be made concerning crack growth in our laboratory model.

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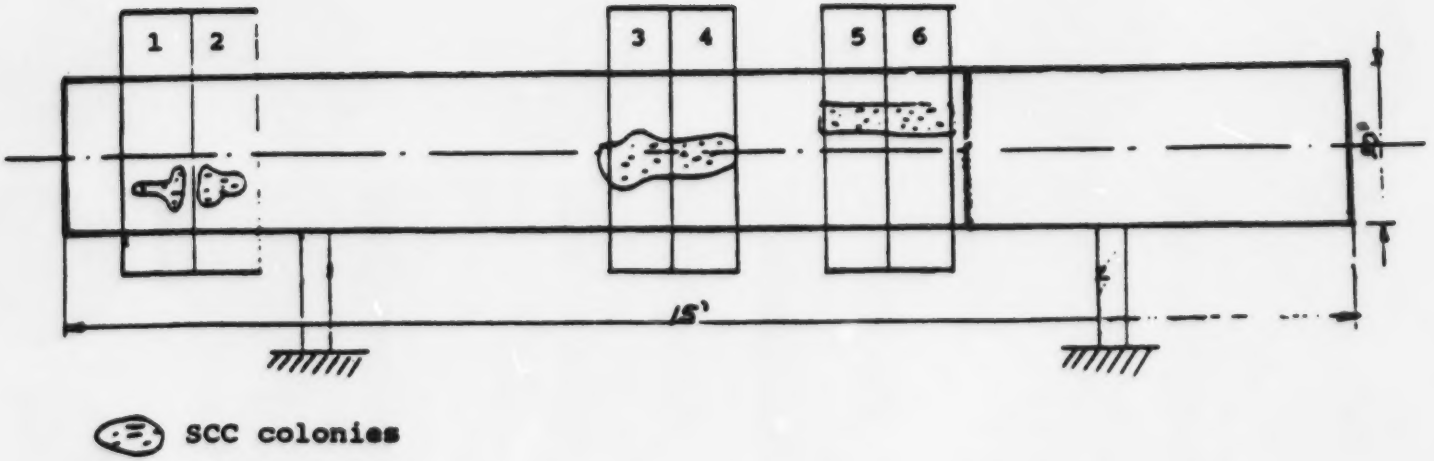


Figure 1. POSITION OF ENVIRONMENTAL CELLS AND NATURAL STRESS CORROSION CRACK COLONIES ON THE TEST PIPE

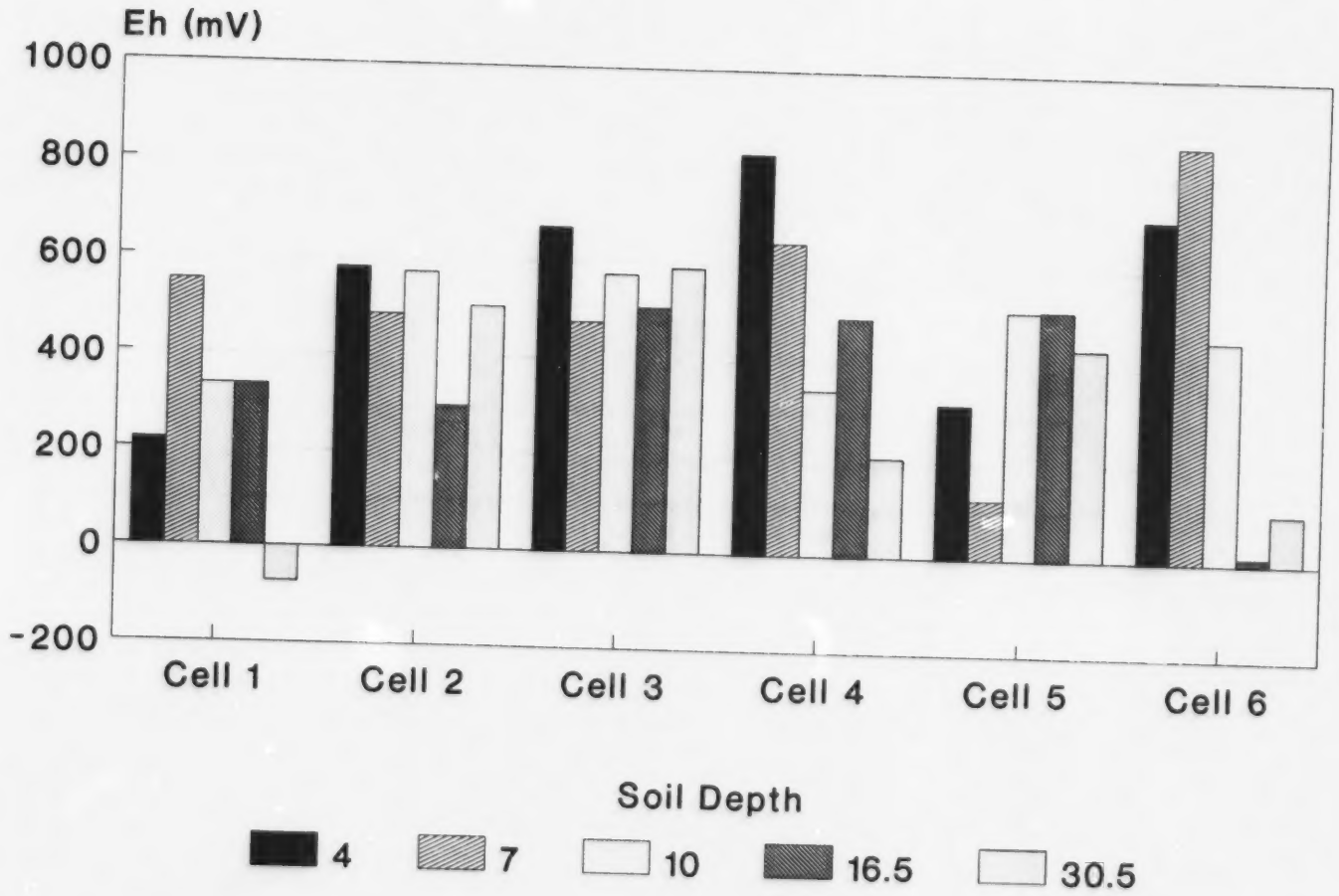


Figure 2. SOIL Eh PROFILE (PERIOD 2)

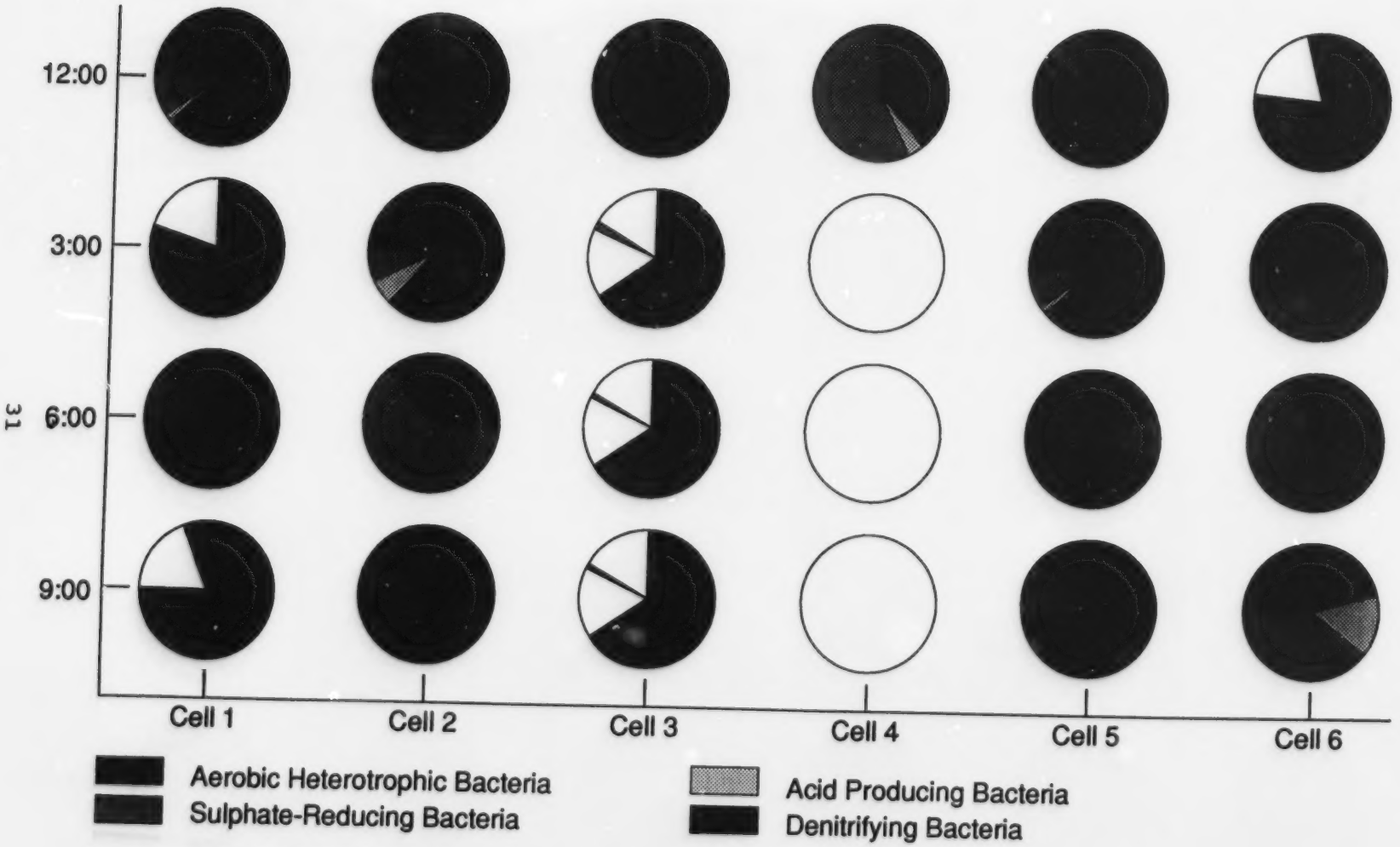


Figure 3. RATIO OF FOUR BACTERIAL METABOLIC GROUPS IN THE TRAPPED WATER AT DISBONDMENT SITES (PERIOD 1)

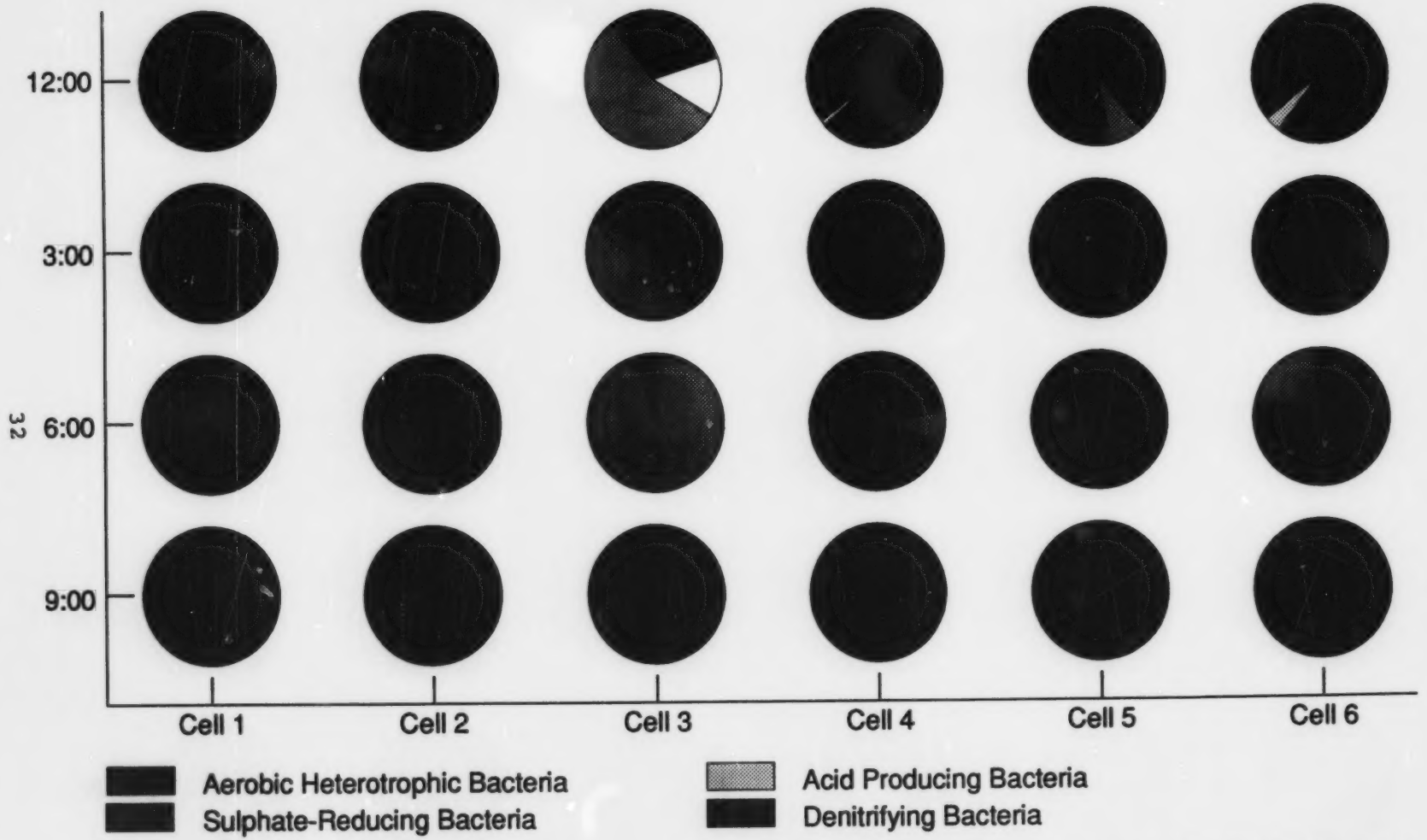


Figure 4. RATIO OF FOUR BACTERIAL METABOLIC GROUPS IN THE TRAPPED WATER AT DISBONDMENT SITES (PERIOD 2)

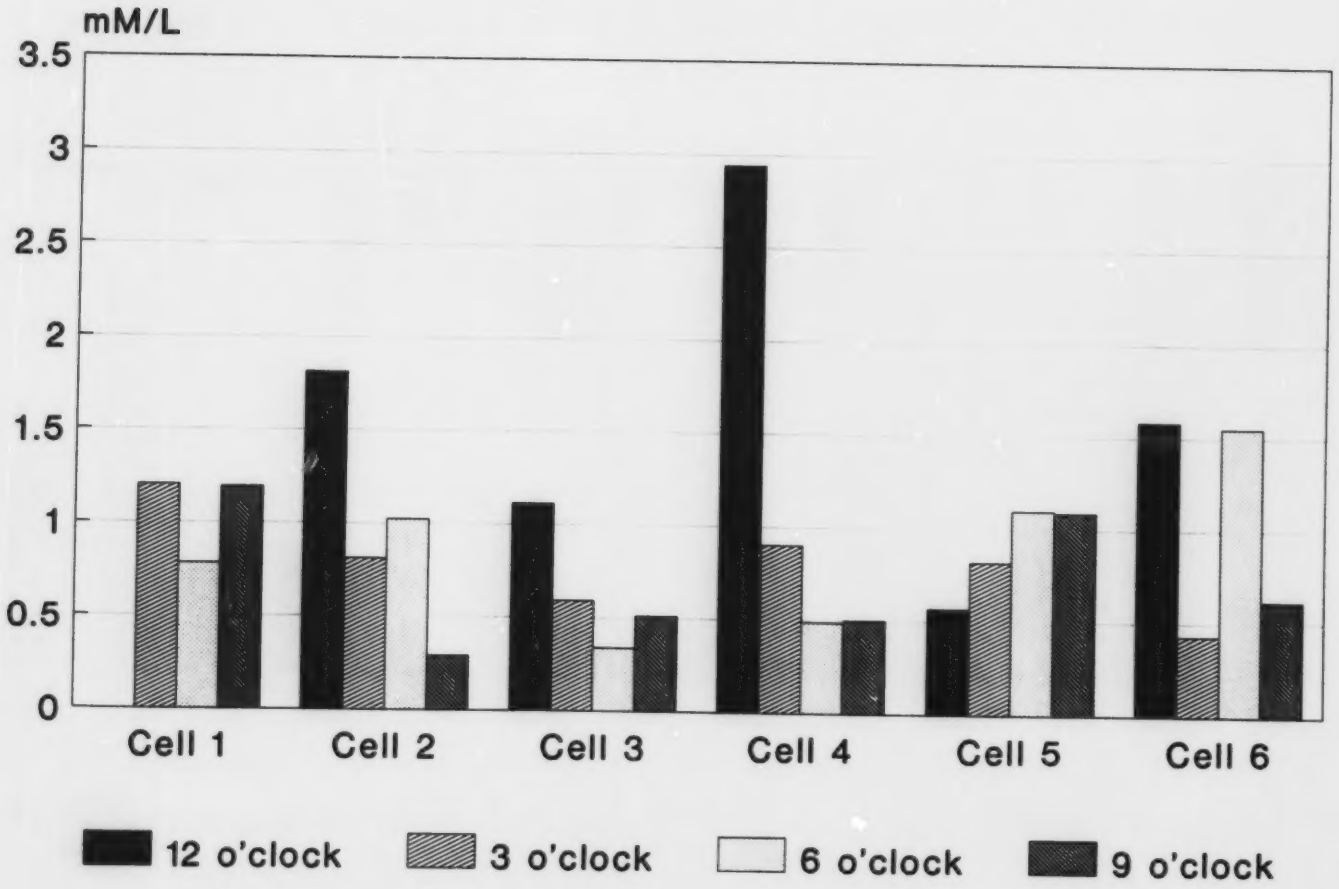
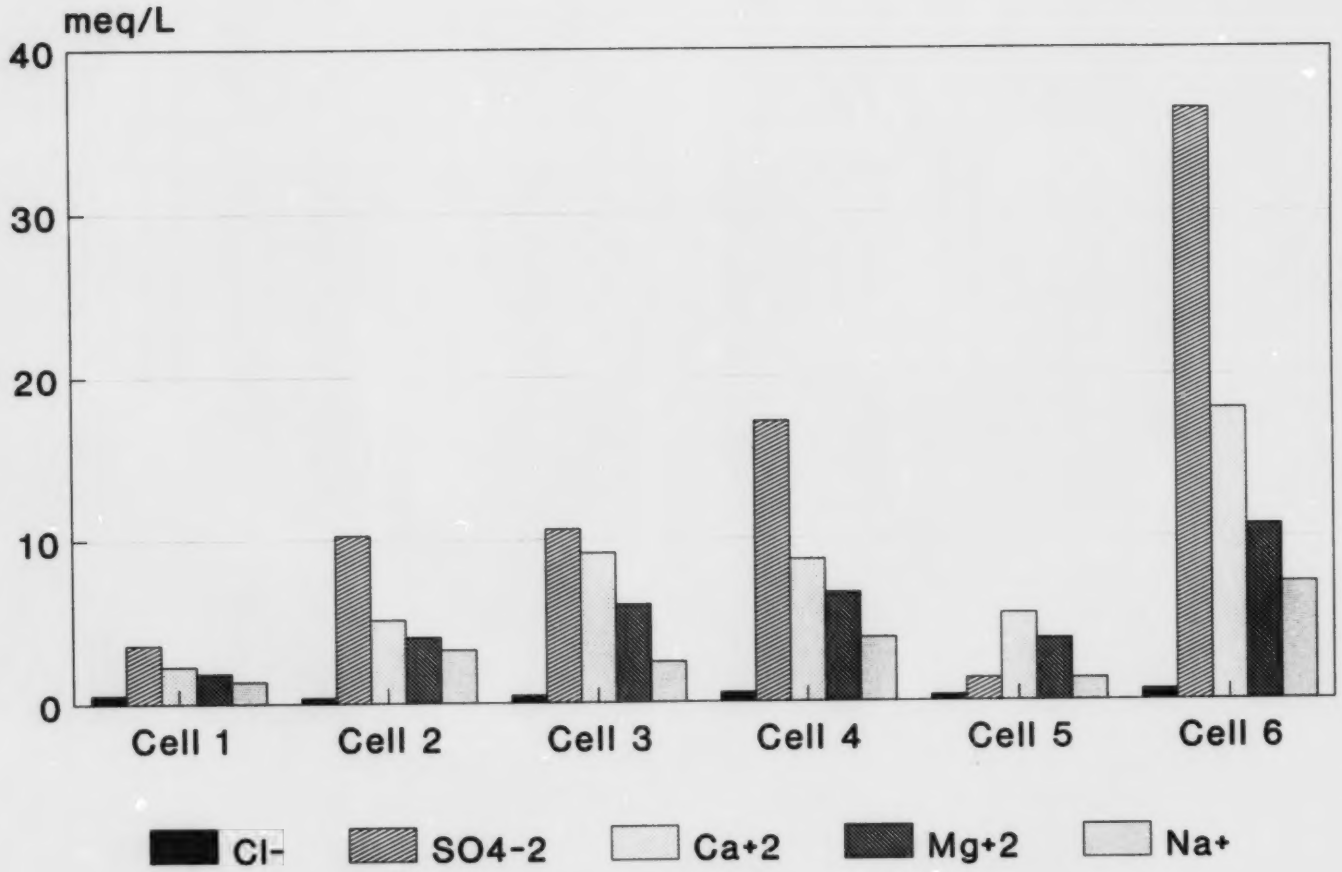


Figure 5. ORGANIC ACID CONCENTRATION IN THE TRAPPED WATER AT DISBONDMENT SITES (PERIOD 2). VALUES ARE THE SUMMATION OF ACETIC, FORMIC, LACTIC, PROPIONIC, PYRUVIC, AND BUTYRIC ACID CONCENTRATIONS



Total iron, K+, Mn+2, NO_2-, NO_3-, PO_4-3, S_2O_3-2 <0.032 meq/L at all sites

Figure 6. ION CONCENTRATION IN THE TRAPPED WATER AT THE DISBONDMENT SITES (PERIOD 2)

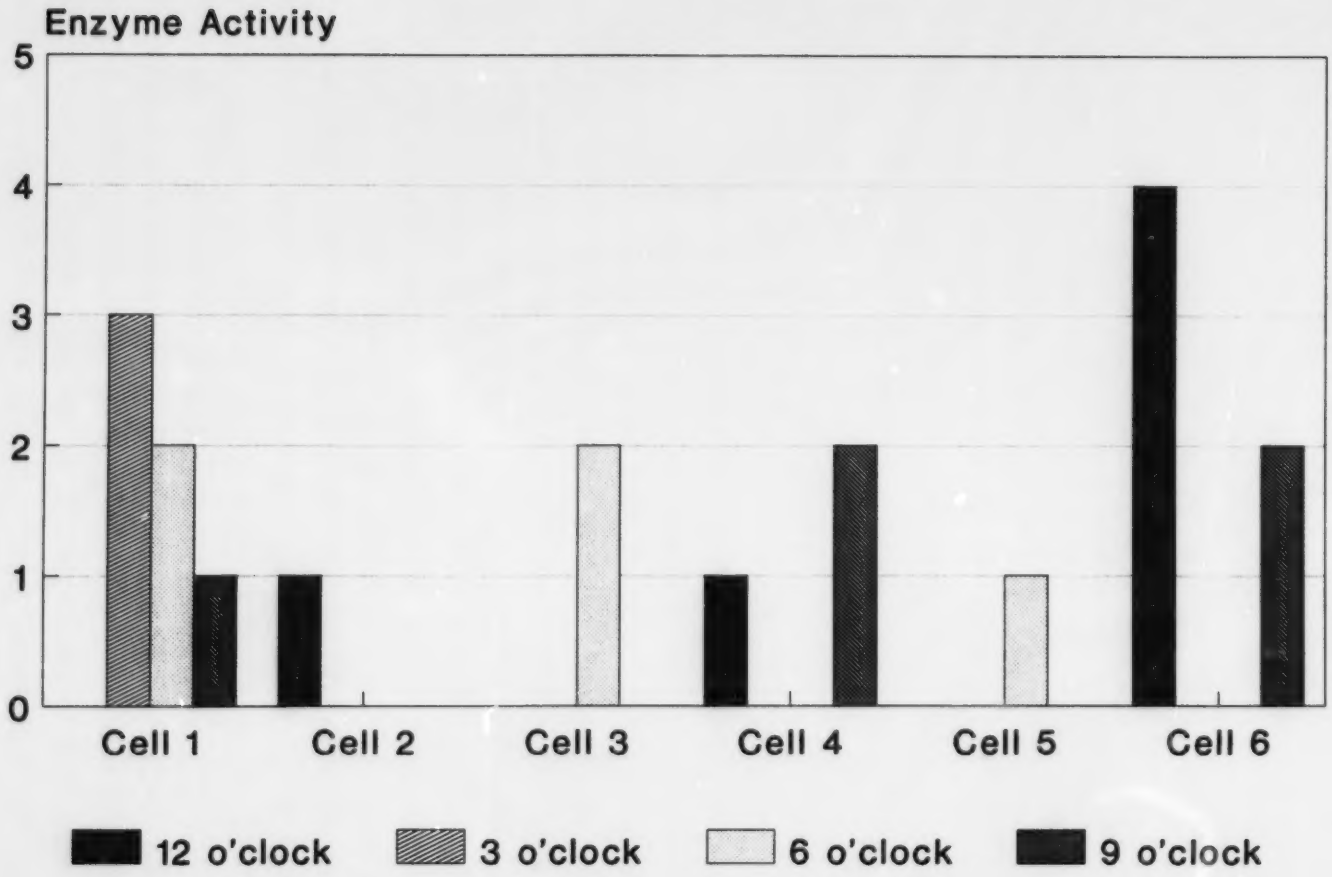


Figure 7. HYDROGENASE ACTIVITY IN THE TRAPPED WATER AT THE DISBONDMENT SITES (PERIOD 1)

APPLICATION OF REVERSE SAMPLE GENOME PROBING TO THE DETECTION OF SULFATE-REDUCING BACTERIA IN OIL FIELD FLUIDS

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ABSTRACT

Since 1988 we have explored gene probe methods to detect the presence of sulfate-reducing bacteria (SRB). Our objective was to characterize the diversity of SRB in samples obtained from Western Canadian oil fields. Initial studies used specific genes cloned from the Desulfovibrio group to detect SRB with either Southern blot or dot blot methods. The gene encoding [NiFe] hydrogenase was found to be a useful probe, since it was present in every species of this group. However, Desulfovibrio represents only one of eight different groups of SRB, that are now recognized and the hydrogenase gene probe failed to detect SRB from the other seven groups. No specific gene probes are currently available for SRB from these other seven groups, but we found that total chromosomal DNA can be used as a specific probe for a given SRB. The diversity of SRB in a sample was most rapidly analyzed by a reverse sample genome probe method (RSGP) in which denatured chromosomal DNAs of relevant SRB standards are first spotted on a master filter, which is then hybridized with labeled DNA extracted from the sample. Analysis of 27 samples with RSGP indicated the presence of at least 16 different SRB in Western Canadian oil fields.

APPLICATION DE LA TECHNIQUE "REVERSE SAMPLE GENOME PROBING" POUR LA DÉTECTION DES BACTÉRIES SULFATO-RÉDUCTRICES DANS LES CHAMPS PÉTROLIFÈRES

RÉSUMÉ

Depuis 1988, nous avons exploré l'utilisation des sondes géniques pour déceler la présence des bactéries sulfato-réductrices (BSR). Notre objectif consistait à caractériser la diversité des BSR dans des échantillons en provenance des champs pétrolifères de l'Ouest canadien. Dans les études initiales, nous avons utilisé des gènes spécifiques clonés provenant du groupe Desulfovibrio pour détecter les BSR, soit par transfert de Southern, soit par "dot blot". On a estimé que le gène codant la [NiFe] hydrogénase constituait une sonde utile puisqu'on le retrouve chez toutes les espèces de ce groupe. Toutefois, le groupe Desulfovibrio ne constitue qu'un seul des huit groupes de BSR actuellement reconnus et la sonde génique de l'hydrogénase ne permet pas de déceler les BSR appartenant aux sept autres groupes. Nous ne disposons pas à l'heure actuelle de sondes géniques spécifiques pour ces sept autres groupes, mais nous avons constaté que l'ADN chromosomique total peut être utilisé comme sonde spécifique pour une BSR donnée. La diversité des BSR dans un échantillon a été analysée très rapidement par une méthode appelée "Reverse sample genome probe" (RSGP) dans laquelle des ADN chromosomiques dénaturés provenant de BSR pertinentes servant d'étalons sont d'abord déposés sur un maître-filtre, puis hybridés avec de l'ADN marqué extrait de l'échantillon. L'analyse de 27 échantillons par RSGP a révélé la présence d'au moins 16 BSR dans les champs pétrolifères de l'Ouest canadien.

APPLICATION OF REVERSE SAMPLE GENOME PROBING TO THE DETECTION OF SULFATE-REDUCING BACTERIA IN OIL FIELD FLUIDS

INTRODUCTION

Testing for SRB in the energy and mining industries is done with metabolic activity tests, monitoring e.g. hydrogen sulfide production or hydrogen consumption. These tests do not distinguish the different groups of SRB, that are now recognized (1, 2). Consequently, it is not clear whether all or only a few selected strains contribute to corrosion and souring problems or are effective in metal ion precipitation in acid mine drainage ponds. Development of tests capable of group or species identification in environmental samples, e.g those based on the principle of nucleic acid hybridization, is therefore of interest. The potential of nucleic acid probes to monitor the presence of microorganisms in environmental samples is well documented (3, 5) and their use in the detection of SRB will be discussed here.

LABORATORY METHODS

Details of the procedures used in our study have been described elsewhere (6, 7). SRB type cultures were obtained from either the NCIMB, DSM or ATCC type culture collections. These were grown on a defined medium described by Pfennig et al. (4), using lactate, propionate, butyrate, benzoate, or acetate as the carbon source. Oil field samples were enriched for SRB by growth in the same medium with either lactate, ethanol, propionate, benzoate, decanoate or acetate as the carbon source. Liquid

culture enriched or colony purified isolates were named according to the carbon source used in their first isolation and an identifying number (e.g.*Lac*4, *Ben*1). DNA was extracted from SRB type cultures and oil field enrichments by a modified Marmur procedure (6, 7). These DNA preparations were analyzed by either of four methods.

Method 1: Southern blot using hydrogenase gene probes. DNAs were digested with the restriction endonuclease *Eco*RI, loaded in the wells of an agarose gel and electrophoresed. This procedure separates the restriction fragments according to size. Bacteriophage λ DNA digested with restriction endonuclease *Hin*dIII, was also loaded to provide size markers of 23.0, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kilobasepairs (kb). Following electrophoresis the DNA fragments in the gel were denatured by alkali treatment and transferred to a nylon or nitrocellulose membrane filter. The filter was incubated with either a labeled [NiFe], [NiFeSe] or [Fe] hydrogenase gene probe. Probes were radiolabeled using [α-^{32}P]dCTP (6, 7). The filter was then washed and dried. The hybridizing bands were identified by autoradiography.

Method 2: Dot blot using hydrogenase or FC11 gene probes. DNAs were heated or treated with alkali and the denatured DNA preparations were spotted on a membrane filter (2 µl, 25 ng/µl; 1 µl is 10^{-6} l and 1 ng is 10^{-9} g). The filter was incubated with a labeled gene probe, obtained from plasmid pHCAL2, pFC11-1 or pFC11-2, containing as inserts the [NiFe] hydrogenase genes of *D. vulgaris* Miyazaki, a 5 kb chromosomal *Eco*RI fragment of FC11 and a 1 kb chromosomal *Eco*RI fragment of FC11, respectively. Following incubation the filter was washed and hybridizing DNAs were identified by autoradiography.

Method 3: Dot blot using chromosomal DNA probe. This procedure was as method 2 except that a chromosomal DNA prepared from a type culture or colony purified oil field isolate was used as the probe.

Method 4: Dot blot using a reverse sample genome probe. The principle of RSGP is outlined in Fig. 1. Denatured chromosomal DNAs prepared for the standards (2 µl, 6 ng/µl) were spotted on a master filter. The standards were SRB isolated previously from oil fields. Some standards were colony purified isolates, but the majority was obtained by liquid culture enrichment of oil field samples. Total DNA was then extracted from an oil field sample, that was usually enriched for SRB by growth on Pfennig's medium. This DNA preparation was in part denatured and spotted on the master filter to provide a positive hybridization control. Another part of the total DNA preparation was labeled and the master filter was incubated with the resulting reverse sample genome probe. Following incubation the filter was treated as in method 2.

RESULTS AND DISCUSSION

Analysis of bacteria in environmental samples by nucleic acid hybridization is usually accomplished by using a species or genus-specific probe, which can either be a deoxyoligonucleotide (15-50 nucleotides) or a cloned gene (0.2 to several kb). The deoxyoligonucleotide is obtained by chemical synthesis and labeled by end-labeling in which e.g. radiolabeled phosphate (^{32}P) is transferred to the 5' end of the deoxyoligonucleotide from [γ-^{32}P]ATP in a reaction catalysed by T4 polynucleotide kinase. The gene is obtained by cloning in a plasmid, which can be amplified biologically. Following biological amplification the plasmid DNA is purified and the

cloned gene separated from the plasmid vector by restriction digestion, and preparative agarose gel electrophoresis. The purified DNA representing the gene is then radiolabeled in a reaction in which the DNA is replicated in vitro using a DNA polymerase and the four deoxynucleotidetriphosphate substrates, one of which is labeled (e.g. with radioactive phosphorous [α-^{32}P]dCTP). Either of two procedures, nicktranslation or random hexamer labeling are used. These will not be detailed here.

Of the eight groups of SRB that are now recognized (1, 2) only group 1, representing the genus *Desulfovibrio*, has been subjected to molecular biological analysis other than 16 S rRNA sequencing. As a result, genes for a number of redox proteins and redox enzymes (e.g. hydrogenases, cytochromes) have been cloned and characterized by nucleic acid sequencing. It is now known that three different hydrogenase enzymes, encoded by three different genes, are present in *Desulfovibrio* spp. These are the [Fe] hydrogenase, the [NiFe] hydrogenase and the [NiFeSe] hydrogenase. Using Southern blots and dot blots (methods 1 and 2) it was found that of 22 *Desulfovibrio* type culture DNAs 13 reacted with the [Fe] hydrogenase and 15 with the [NiFeSe] hydrogenase probe, whereas all 22 reacted with the [NiFe] hydrogenase probe. The latter represents a good genus-specific probe: positive reaction of a DNA preparation indicates the presence of *Desulfovibrio* spp. Importantly, we found that DNAs isolated from SRB type cultures of the other seven groups did not react with the *Desulfovibrio* hydrogenase gene probes. The application of this finding to samples obtained from Nova-Husky oil fields is shown in Fig. 2. Of 20 samples analyzed, 12 reacted positively, whereas 8 showed no hybridization with either the [NiFe] or the [Fe] hydrogenase gene probes. This negative results indicates that these 8 samples did not contain SRB from the genus

Desulfovibrio. Since in a Southern blot DNA fragments are separated according to size (Fig. 2) it is possible to indicate the number of different *Desulfovibrio* species present by comparing the sizes of the hybridizing bands in the positively reacting samples. The results indicate the presence of 5 different *Desulfovibrio* species (Table 1). Interestingly, samples taken from different production facilities at a given site (e.g. Table 1, site II: samples 6-8) showed differences in the *Desulfovibrio* species present. This degree of differentiation requires use of Southern blotting (method 1). If the samples were to be analyzed by dot blotting method 2, only the presence (positive signal) or absence (negative signal) of *Desulfovibrio* species would be detected.

Although the application of methods 1 and 2 is useful for detection of *Desulfovibrio* species its fails to detect the other SRB groups. In our efforts to develop DNA probes for non-*Desulfovibrio* SRB we considered a colony purified isolate obtained from a Pembina oil storage tank connected to the Interprovincial Pipeline, Edmonton, Alberta. This isolate, FC11, did not react with the *Desulfovibrio* hydrogenase probes (Fig. 3D). A number of isolates (FC9-FC17) were obtained from the same location, which were thought to be similar or identical to FC11 on the basis of microbiological criteria, e.g. appearance in the microscope. Our strategy for the isolation of a DNA probe for FC11 was to: (i) digest chromosomal DNA from FC11 with *Eco*RI, (ii) clone the *Eco*RI fragments at random in vector pUC8 and (iii) check the specifity of the FC11 DNA inserts of the resulting plasmids in detecting FC9-FC17 and not other SRB. Two plasmids pFC11-1 and pFC11-2 were isolated from steps (i)-(ii), with FC11 DNA inserts of 5 kb and 1 kb, respectively. Use of the 5 kb pFC11-1 insert as a probe in a dot blot assay (method 2) gave the results shown in Fig. 3A. The probe

specifically hybridized with FC9-FC17, but not with other SRB. Use of the 1 kb EcoRI probe from pFC11-2 gave similar results (Fig. 3B). It thus appeared very easy to isolate fragments of the FC11 chromosome that could be used for its specific detection and the possibility to use the entire FC11 chromosome as a specific probe in dot blot assays (method 3) was therefore investigated. The results in Fig. 3C indicate that a FC11 chromosomal DNA probe does not exhibit significant crosshybridization with the chromosomal DNAs of other SRB. Similarly, use of chromosomal DNA probes from SRB type cultures, e.g. *Desulfovibrio salexigens*, *Desulfotomaculum orientis* and *Desulfovibrio thermophilus*, indicated selective hybridization only to self or very similar SRB genotypes.

The extensive genetic diversity of SRB, derived by Devereux et al. on the basis of 16S rRNA sequencing (1, 2), thus endows different SRB with chromosomes that exhibit little or no crosshybridization. The chromosomal DNA of a given sulfate-reducing bacterium can thus be used as a sensitive probe for its presence in the environment. Of course use of chromosomal DNA probes does not allow differentiation of highly homologous SRB, e.g. subspecies of a given genotype. We therefore refer to a single or a collection of homologous organisms as a standard. Different standards have chromosomal DNAs that show little or no crosshybridization. Using this criterion of uniqueness of hybridization of chromosomal DNA, we have been able to isolate and define 21 different SRB standards from Alberta oilfields (Table 2). These represent SRB first isolated on lactate (8 standards), ethanol (1 standard), benzoate (2 standards), decanoate (3 standards), propionate (4 standards) and acetate (3 standards). Of these 21 standards, 9 belong to the genus *Desulfovibrio* in view of the hybridization

of their DNA with *Desulfovibrio* specific hydrogenase gene probes (Table 2).

The composition of a sample can thus be defined by probing DNA isolated from the sample with dot blot method 3, in which DNA from one of the standards is used as a probe. This requires 21 separate incubations of 21 dot blots containing the denatured sample DNA, if one wants to probe for the presence of all 21 standards. We considered that, alternatively, denatured DNA isolated from the standards could be spotted on a filter and the resulting master filter hybridized with labeled sample DNA (method 4). We refer to this method as reverse sample genome probing, because the sample-probe relationship is reversed relative to conventional dot blot hybridization assays (methods 2 and 3). In RSGP, of which the principle is outlined in Fig. 1, one qualitatively finds the primary bacterial standards present in the sample in a single step. However, it must be realized that development of an appropriate master filter requires a considerable research effort. It is not sufficient in the case of SRB to spot DNA obtained from genotypes deposited in culture collections. Indeed, most of the appropriate standards must be isolated from the field (Table 2). Also, we have found that SRB from different environments (e.g. oil fields, waste waters, acid mine drainage ponds) are so diverse that master filters targeted for each environment must be developed.

Application of RSGP to the analysis of SRB in oil field samples is shown in Fig. 4. In all cases (Fig. 4 A, B, C) the denatured sample DNA preparation was also spotted to provide a positive hybridization control. RSGP thus indicates that the samples in Figs. 4A, 4B and 4C contain primarily standards *Lac4*, *Lac5* and *Ben1*, respectively. An analysis of 27 oil field samples with RSGP gave the results indicated in Table 3. It is clear

that a variety of SRB is present in these samples. The data in Table 3 represent only a start in the analysis of the complexity of the population of SRB in Alberta oil fields, since master filters with only 21 standards were used in the analysis. The current version of the master filter in use in our laboratory contains upto 47 SRB standards. The application of RSGP in the analysis of the microbial populations in Alberta oil fields shows that the types of SRB found are quite variable. An understanding of this variability is needed in the planning and evaluation of biocide tratment schemes.

ACKNOWLEDGEMENTS

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Table 1 - Analysis of oilfield samples for the presence of SRB from the genus *Desulfovibrio* with hydrogenase gene probes.

Sample no.[a]	Site	Production facility	*Desulfovibrio* species[b]
1	I	Testloop line 1	-
2	I	Testloop line 2	-
3	I	Testloop line 3	-
4	I	Testloop line 4	-
5	I	Testloop line 5	-
6	II	Washtank	D1
7	II	Water plant 1	D1
8	II	Water plant 2	D1,D2,(D5)
9	III	Washtank	D1
10	III	Water plant	D1
11	IV	Water plant	D3
12	V	Free water knockout	D1,D2
13	V	Water plant	D1,D2
14	VI	Free water knockout	D4
15	VI	Water plant	D1,D3
16	VII	Water plant	-
17	VIII	Free water knockout	D4
18	VIII	Water plant	D1
19	IX	Free water knockout	-
20	IX	Water plant	-

[a] All samples were obtained from Nova-Husky oilfields in Alberta and cultured on lactate-sulfate medium.

[b] Designations of different *Desulfovibrio* species are on the basis of the hybridization patterns of DNA isolated from the samples with probes for the [NiFe] and [Fe] hydrogenase genes (Fig. 2). -, Failure to hybridize.

Table 2 - Standards for RSGP analysis isolated from oil field and waste water samples[a].

Isolate	Standard	Colony purified	Cross hybridization	[NiFe]	[Fe]	Identification	Reference or number
1[b]	*Lac1*	yes	**Lac2**	+	+	*D. vulgaris* subsp. *oxamicus* Monticello	7
2[b]	*Lac2*	yes	**Lac1**	+	-	*D. vulgaris* subsp. *oxamicus* U of A	7
3	*Lac3*	yes	-	+	+	*D. desulfuricans* G200	7
4	*Lac4*	no	*Lac5*	+	-	*Desulfovibrio* sp.	6
5	*Lac5*	no	*Lac4*	+	-	*Desulfovibrio* sp.	6
6	*Lac6*	no	-	+	+	*Desulfovibrio* sp.	6
7	*Lac7*	yes	-	-	-	FC11, unidentified	6
8	*Lac8*	no	-	+	+	*Desulfovibrio* sp.	6
9	*Lac10*	no	-	+	+	*Desulfovibrio* sp.	6
10	*Eth1*	no	-	+	-	*Desulfovibrio* sp.	6
11	*Ben1*	no	-	+	-	*Desulfovibrio* sp.	6
12	*Ben2*	no	-	-	-	unidentified	6
13	*Dec1*	no	*Dec3*	-	-	unidentified	6
14	*Dec2*	no	-	-	-	unidentified	6
15	*Dec3*	no	*Dec1*	-	-	unidentified	6
16	*Pro1*	no	*Pro3*	-	-	unidentified	6
17	*Pro2*	no	-	-	-	unidentified	6
18	*Pro3*	no	*Pro1*	-	-	unidentified	6
19	*Pro4*	no	-	-	-	unidentified	6
20	*Ace1*	no	-	-	-	unidentified	6
21	*Ace2*	no	-	-	+	unidentified	6
22	*Ace3*	yes	-	-	-	*Desulfobacter hydrogenophilus*	6

[a] The standards are named by the first three letters of the carbon source used for their first isolation on medium defined by Pfennig et al. (4) and an identifying number. Carbon sources used are lactate, ethanol, propionate, benzoate, decanoate and acetate. Weak cross-hybridization of a standard with others (strong hybridizations are in bold), as well as hybridization (+) or lack of hybridization (-) with [NiFe] and [Fe] hydrogenase gene probes from *Desulfovibrio* spp. are indicated.

[b] In view of strong crosshybridization *Lac1* and *Lac2* represent the same standard *Lac1,2*.

Table 3 - Identification of SRB in samples from oil-fields with RSGP, using master filters containing the standards listed in Table 2[a].

Sample no.	Sample	Identification[b]
1	Nova Husky #1A	*Lac5;Ben1;Dec3*
2	Nova Husky #1B	*Lac1,2;Lac4;Lac5*
3	Nova Husky #2	*Lac5;Ben1*
4	Nova Husky #3A	*Lac4;Lac5;Ben1;Dec3;Pro2*
5	Nova Husky #3B	*Dec3*
6	Nova Husky #4	*Lac4;Lac5;(Ben1)*[c]
7	Nova Husky #5	*Lac4;Lac5;Ben1;Pro1;Dec3*
8	Nova Husky #6	*Lac4;Lac6;Ben1;Lac7*
9	Nova Husky #7	*Lac5;Lac4;Ben1;Ace1;(Lac1,2)*
10	Nova Husky #8	*Lac4;Lac10*
11	Nova Husky #10	*Lac10*
12	Nova Husky #10A	*Ben1;Pro3*
13	Nova Husky #10B	*Lac5*
14	Shell #1A	*Lac4;Lac3;*
15	Shell #1B	*Lac4*
16	Shell #2A	*Lac6*
17	IPL #21	*Lac3;Lac8*
18	IPL #22	*Lac1,2;Lac8*
19	IPL #27	*(Lac1,2;Lac8;Dec1)*
20	Virginia Hills #1	*Lac4;Lac6*
21	Virginia Hills #2	*Lac4;Lac6;Ben1;Dec1*
22	Virginia Hills #6	*Lac6*
23	House Mountain #1	*Lac4;Lac6;Ben1*
24	House Mountain #2	*Lac4;Lac6;Ben1;Dec3*
25	House Mountain #3	*Lac4;Lac6;Ben1*
26	House Mountain #4	*Lac6*
27	House Mountain #6	*Lac6;Pro4*

[a] The samples were analyzed following growth on Pfennig's medium using one of six carbon sources: lactate, ethanol, benzoate, decanoate, propionate or acetate. The resulting DNA preparations (maximally six, if the sample grew on all six carbon sources) were analyzed by RSGP. The results obtained have been combined in the stated composition of the sample.

[b] *Lac1,2* is a combination of standards *Lac1* and *Lac2* (see Table 2)

[c] Parentheses indicate very weak signals.

Analysis of total DNA isolated from an oil field or other environmental sample with RSGP using a master filter with 12 standards:

- **Isolate total DNA from sample (S).**

- **Spot denatured DNA (S) on master filter containing denatured DNAs from relevant bacterial standards 1-12.**

- **Label S to S*. Incubate S* with master filter.**

- **Develop to see hybridization of S* with master filter.**

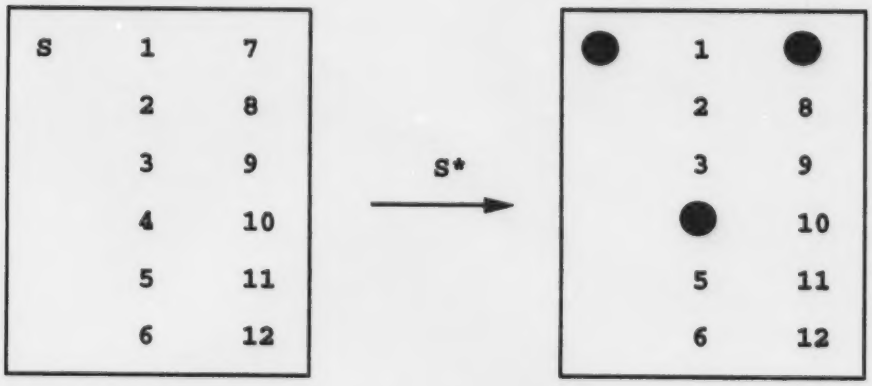

Fig. 1 - Principle of RSGP. The sample S contains standards 4 and 7. Any number of standards can be used.

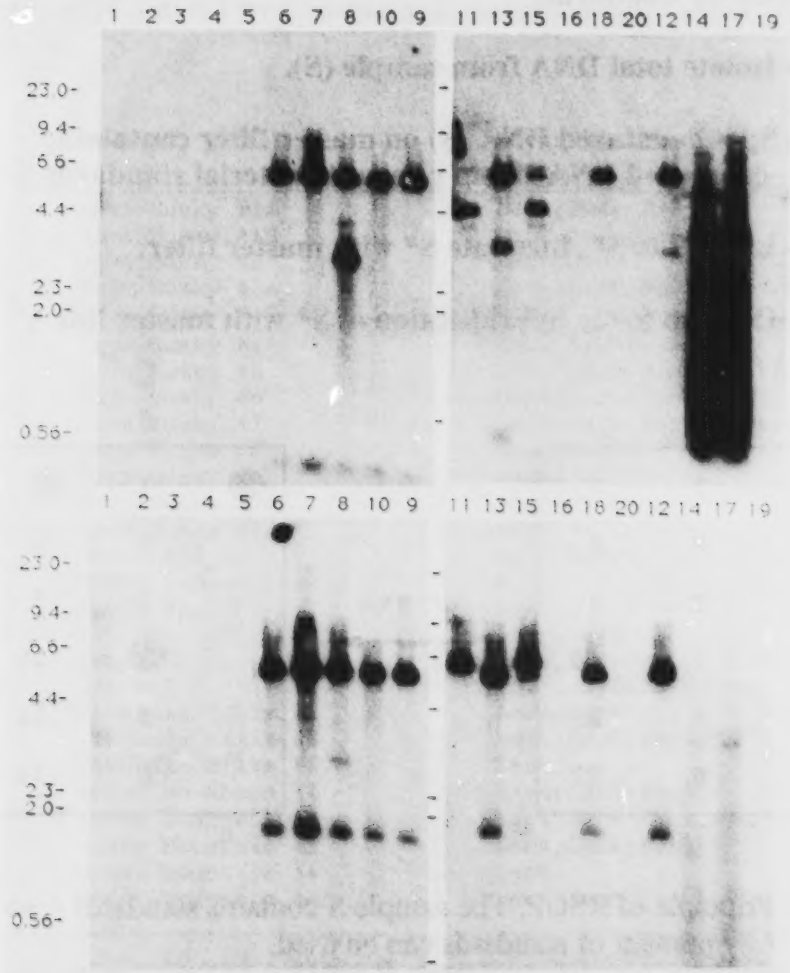

Fig. 2 - Detection of *Desulfovibrio* species in samples from the oil-field production facilities listed in Table 1. Purified total DNA was digested with *Eco*RI and Southern blotted (method 1). The blot was incubated with either the radiolabeled [NiFe] hydrogenase gene probe (top) or the [Fe] hydrogenase gene probe (bottom). The numbers on the left are molecular weight standards (kilobasepairs). Reprinted with permission of Applied and Environmental Microbiology.

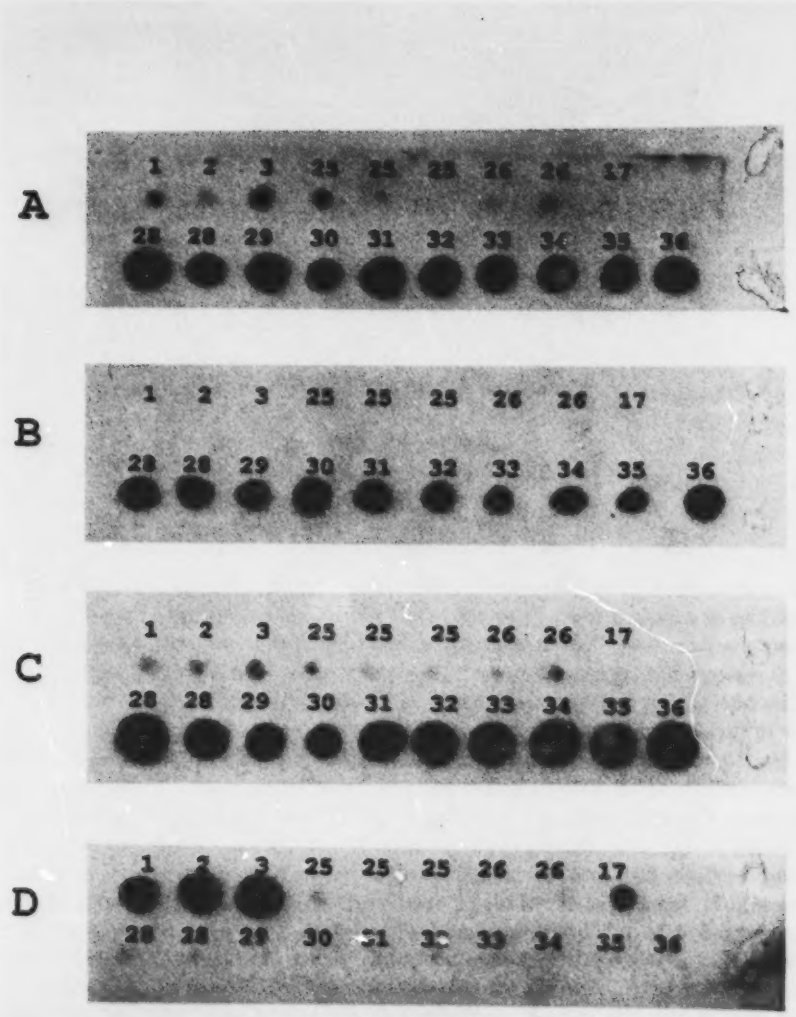

Fig. 3 - Hybridization specificity of FC11 DNA probes. Denatured DNA's (2 µl, 25 ng/µl) from type cultures [1-3] *Desulfovibrio vulgaris* strains; [17] a *Desulfovibrio desulfuricans* strain; [25 and 26] *Desulfotomaculum ruminis* strains and [28 - 36] unidentified colony purified field cultures FC9-FC17 were spotted on the filters. These were incubated with the following nick-translated probes: (A) 5 kb *Eco*RI insert from pFC11-1, (B) 1 kb *Eco*RI insert from pFC11-2, (C) chromosomal DNA from FC11 and (D) the 1 kb *Eco*RI fragment containing part of the structural genes of [NiFe] hydrogenase from *D. vulgaris* Miyazaki. Reprinted with permission from Applied and Environmental Microbiology.

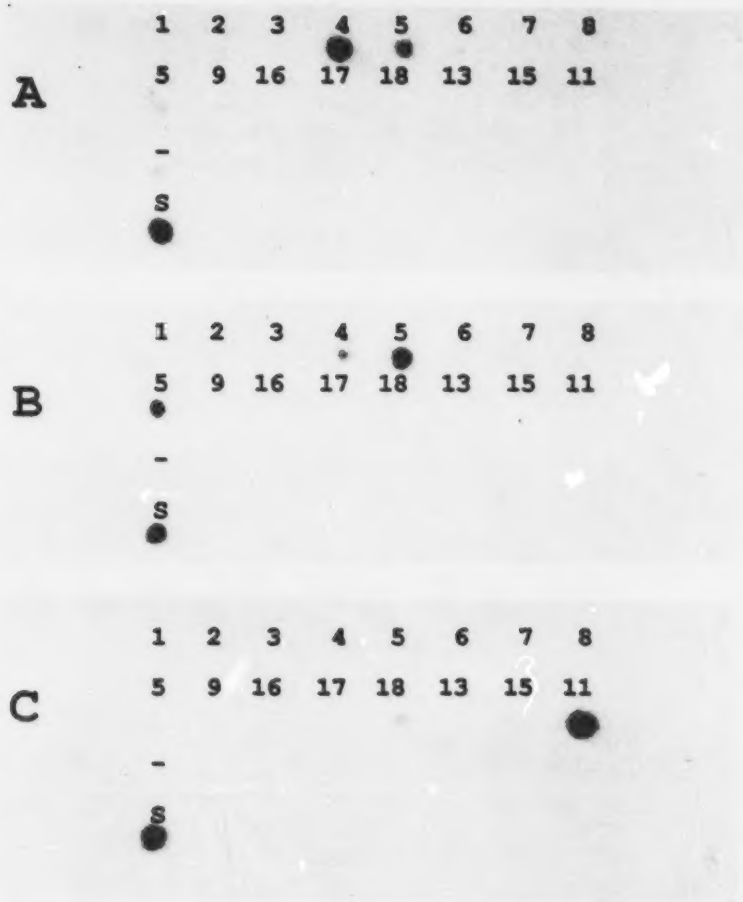

Fig. 4 - Analysis of DNA from field samples by RSGP. Denatured DNAs (2 µl, 6 ng/µl) from a set of 15 standards were spotted on the filters. The numbers for the standards correspond to those in Table 2. The sample DNA preparation (S) to be analyzed (2 µl, 6 ng/µl; was spotted in the lower left-hand corner of filters A, B and C. The undiluted sample DNA preparation (3 µl, 10-100 ng/µl) was then radiolabeled by nick-translation and the filters were incubated with the resulting reverse genome probe. Reprinted with permission from Applied and Environmental Microbiology.

BIOTREATMENT OF WASTE OIL AND SLUDGES

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In recent years the petroleum industry has substantially reduced the volume of hydrocarbon wastes generated annually. However, there is still a significant amount of hydrocarbon waste which cannot be reclaimed and needs to be disposed of. Conventional disposal methods have relied on landfarming but this is a slow process dependent on weather and location. Biological treatment of hydrocarbon wastes in a bioreactor system appears to be a competitive disposal alternative. Following successful degradation of waste oil from conventional oil operations where 74% - 78% of the oil was degraded over an 11 - 15 month period, interests were directed to heavy oil wastes. Effective degradation of 44% of heavy oil was achieved in a bioreactor over a five month operation period. Biodegradation of the oil correlated with the presence of oil degrading microbes capable of degrading complex molecules containing up to four fused aromatic rings. The residue consists of non oily Oclansorb bearing relative water-insoluble hydrocarbons which are non-volatile and consists primarily of asphaltenes. This material does not support microbial growth and is therefore not readily biodegradable. The residue may be remixed with additional oily sludge and subjected to at least one additional biodegradation cycle in the bioreactor. Ultimate disposal of the residue by landfarming may require prolonged treatment for complete destruction of hydrocarbon but the process would reduce the initial load on such a facility, remove volatiles responsible for odors or VOC emissions associated with such operations and ultimately provide revegetation benefits for the site. A second disposal option for the residue may be a landfill once oily components and prohibited compounds and toxics have been removed by biodegradation in the bioreactor.

INTRODUCTION

Sludges generated by the oil industry can contain up to 40% - 60% oil (Table 1) (1). Due to both economical and environmental constraints over the last few years, the volume of waste oil has been considerably reduced due to improved operating procedures and reclamation methods such as centrifugation to separate oil from water in slop oils. However, there will always be some material which will have to be disposed of eventually. Traditionally, the disposal method of choice has been landfarming and there are landfarms at nearly every major oil refinery in the country (2). In a landfarm operation a site at the refinery is dedicated to the process and the waste oil and oily sludges are ploughed into the soil. Nitrogen and phosphorus nutrients are added to the soil to foster microbial activity to degrade the hydrocarbons. However, landfarms have their limitations (3). They are at the mercy of the length of the summer season; this is especially critical in the northern climates where summer lasts a few months. There are also limitations on loading rates, concerns about volatiles escaping from the landfarm and fears of hydrocarbons and heavy metals leaching from the site into the groundwater. This latter is a particular concern not

TRAITEMENT BIOLOGIQUE DES HUILES ET DES BOUES RÉSIDUAIRES

RÉSUMÉ

Au cours des dernières années, l'industrie pétrolière a réduit de façon appréciable le volume des résidus hydrocarburés produits annuellement. Cependant, il existe encore une quantité importante de résidus hydrocarburés irrécupérables dont il faut se débarasser. Les méthodes d'élimination traditionnelles reposent sur l'épandage des boues sur le sol, mais ce processus est lent et il dépend des conditions météorologiques et du site. Le traitement biologique des résidus hydrocarburés dans un bioréacteur semble être une option d'élimination concurrentielle. Suite au succès obtenu dans la dégradation des huiles résiduaires provenant des opérations classiques d'exploitation (dégradation de 74 à 78% des huiles sur une période de 11 à 15 mois), on s'est intéressé aux résidus d'huile lourde. La dégradation efficace de 44% de l'huile lourde a été réalisée dans un bioréacteur sur une période d'exploitation de cinq mois. On a établi une corrélation entre la biodégradation de l'huile et la présence de microbes produisant la dégradation de l'huile capables de décomposer des molécules complexes contenant jusqu'à quatre noyaux aromatiques fusionnés. Le résidu est de l'Oclansorb non huileux renfermant des hydrocarbures relativement insolubles dans l'eau qui sont non volatils et sont des composés principalement d'asphaltènes. Ce produit ne soutient pas la croissance microbienne et n'est donc pas facilement biodégradable. Le résidu peut être mélangé de nouveau avec une boue huileuse additionnelle et soumis à au moins un cycle de biodégradation de plus dans le bioréacteur. L'élimination finale du résidu par épandage sur le sol peut nécessiter un traitement prolongé visant à produire la destruction complète des hydrocarbures, mais le procédé permet de réduire la charge initiale, d'éliminer les substances volatiles qui causent les odeurs ou les émissions de composés organiques volatiles liées à ces opérations et en fin de compte offre des avantages sur le plan de la restauration du couvert végétal du site. Une seconde option d'élimination du résidu peut être le remblayage une fois que les constituants huileux et les composés et agents toxiques interdits ont été éliminés par biodégradation dans le bioréacteur.

only because groundwater migrates underground and can transport contamination well beyond the landfarm site but also because groundwater is increasingly becoming a source of potable water worldwide (4). Added to this is the recurring threat by the EPA in the United States to discontinue landfarming as a disposal technique (5). Clearly, alternatives are required.

EXPERIENCES WITH BIOTREATMENT

Biotreatment of waste oil and oily sludges has been traditionally performed in landfarms. Studies performed at Guelph University to determine methods of accelerating biodegradation rates in landfarms employed controlled fertilization rates and increased tilling to effect increased degradation (6). However this is a labor intensive process which the refinery does not want particularly. Bioreactor systems for waste oil degradation have been proposed. A Dutch firm, Maurik, currently sells a technology in Europe which appears to be based on the increased tillage - landfarming concept applied in an enclosed space and employing some means of trapping/controlling volatiles.

An enclosed bioreactor system was studied by Dibble at Texaco for the American Petroleum Institute (7). In this system the oil was first absorbed onto rice hulls. During operation rice hulls were more degraded than oil. Oil losses appeared to be due to a combination of microbial action and volatilization caused by the heat generated by microbial action on the rice hulls. While a bioreactor system appears to be feasible, an oil absorption matrix which is inert to biodegradation would be preferred.

BIOTREATMENT OF HYDROCARBONS AT NHRC

At NHRC we first investigated accelerating oil degradation by adding a heat treated peat product, Oclansorb, sold by Hi Point Industries to test plots at a landfarm. This material stimulated oil degradation and growth of oil degrading microbes (Figure 1) (3,8). Peat is the residue of plant material which has already been subject to microbial effects in a peat bog and hence should be relatively refractile to further biodegradation. It therefore appeared to be worthwhile candidate for the inert matrix for oil degradation. We first attempted to degrade waste oily sludge in a bioreactor after the hydrocarbons were first absorbed onto the Oclansorb. Oclansorb is filled with small pore spaces (Figure 2) and absorbs up to 5 times its weight in oil. After absorption, the oil in no longer visible and can be barely detected by smell. This bioreactor was located at Husky Oil's refinery at Prince George, B.C. The microbial numbers rapidly increased in the bioreactor and were associated primarily with the solid matrix of the bioreactor rather than with the leachate water (Figure 3). The microbes actually formed a biofilm on the surface of the peat particles (Figure 4). Although the bioreactor was started in the fall of the year, the reactor partially self-heated until the ambient temperature reached less than minus 20 degrees Centigrade when the reactor froze. However, the microbes were not killed by freezing but remained viable over the winter. Following the spring thaw microbial activity resumed (Figure 3). After 11 -14 months, 74% -78% of the oil had been degraded (Table 2). Slightly more oil was degraded in the top of the bioreactor than in the bottom and this may be related to nutrient limitations or to anaerobic conditions in the bottom of the bioreactor.

The oil used at Prince George is a light, waxy crude and the sludge consisted of primarily

saturate/straight chain molecules averaging in size from C-30 to C-35 (Figure 5). Here in Alberta, there is a lot of heavy oil such as at Lloydminster and in the tarsands. Heavy oil is a more heterogeneous mixture containing complex molecular structures (Figure 6). However, microbial degradation of heavy oil does take place but at a slower rate than conventional oil (9). It was therefore necessary to determine how effective a bioreactor would be at treating heavy oil sludges.

A small pilot bioreactor was constructed at Husky Oil's Lloydminster refinery in 1989. The bioreactor contained two sections. Heavy oil sludge from an API separator was absorbed to the Oclansorb before it was placed in one section of the bioreactor while oil impregnated diatomaceous earth used to break emulsions in a filter press trial was mixed with Oclansorb and placed in the other section of the bioreactor. The bioreactor was also seeded with an oil degrading microbial culture maintained at NHRC. Microbial numbers, activity and oil content were monitored over a 21 month period. Microbial numbers remained stable over several months operation (Figure 7). These organisms were capable metabolizing increasingly complex hydrocarbon molecules containing up to four or five rings (Figure 7). Few organisms were found capable of growing on asphaltenes. Microbial growth on asphaltenes is rare (10).

The amount of oil degraded in the bioreactor was 32% - 34% in the API Separator sludge side and 68% - 75% in the Filter Press Aid side (Figure 8). The relative content of asphaltene in the residual oil increased (Table 4) and correlates with the lack of organisms capable of growing on asphaltene. However, the fact that asphaltenes do not accumulate in landfarms in levels proportional to their loading rates, based on their contents in oily sludges, is indirect evidence of their biodegradation (9). It may require a very long time period to effect significant degradation of this oil fraction. Treatment of the residue with oxidizing agents like peroxide, permanganate or Fenton's reagent (11) might make it more susceptible to biodegradation. Degradation rates in the bioreactor are superior to those achieved in a conventional landfarm operation (Figure 9).

The leachate water contained no PAHs and no heavy metals (Table 5). It does not pose an environmental hazard and can therefore be disposed of in normal refinery wastes or by deep well injection. The solid matrix from the bioreactor is non- flammable and contains low levels of heavy metals which are not leachable (Table 5). It could be placed on a landfarm with the resultant seeding of the landfarm with oil degrading microbes. This material could also help stimulate revegetation of the landfarm. Previous studies showed that use of Oclansorb in a landfarm test plot stimulated microbial degradation of the oil and also stimulated plant regrowth (8). However, low degradation rates for asphaltenic materials and its high content in the bioreactor matrix argues against continued application to a landfarm in order to avoid overloading the site with this oil fraction. Another disposal option is to landfill the matrix. The rules on landfilling are complex and may vary with different jurisdictions. However, low metals contents (Table 5), its non-flammability and lack of volatiles make it a candidate for landfilling. After leachate and toxicity tests were performed on the bioreactor matrix to establish that it is non toxic, the matrix was acceptable to the Lloydminster landfill. This has established that it is possible to biodegrade the waste oil in a bioreactor and landfill the non-biodegradable residue.

A study on the economics of the bioreactor was performed by NOVA's Technology Management Office. It suggested that to be competitive with other disposal options such as the Taciuk Process and Mobil's coking technology the spent matrix would have to be recycled 3 - 4 times and the

sludge loadings would have to be 2:1 sludge/Oclansorb ratio. To determine if this were possible we performed a series of lysimeter studies in the laboratory. The lysimeters operated in a manner similar to the bioreactor. Oclansorb impregnated sludge was placed in the columns and the leachate recycled through the use of a pump.

Sludge:Oclansorb loading ratios of 2:1 were possible and permitted high levels of oil degrading microbes (Table 6) and oil degradation (Table 7). A 2:1 loading ratio represented 1.67:1 oil/Oclansorb ratio. The heavy oil API Separator sludge at Lloydminster contains up to 50% oil so at least 3.3:1 sludge/Oclansorb loading ratios are possible. Another heat treated peat other than Oclansorb also permitted growth of high numbers of oil degraders and oil degradation (Table 6, 7). Conversely, a chemical absorbent such as Fuller's Earth did not obtain much oil degradation (Table 7) probably due to lack of flow in the column. The recycled Oclansorb from the Lloydminster bioreactor functioned well in the lysimeter column and achieved 11% -15% biodegradation (Table 7). Combination of the increased loading rates and the recyclability of the spent matrix could make the bioreactor competitive with other technologies. The bioreactor is ideally suited to treatment/disposal of low volume, high oil content wastes.

In conclusion, the effectiveness of biodegradation of a range of waste oily sludges in a bioreactor has been demonstrated. Use of a bioreactor has several advantages over a conventional landfarm. The bioreactor is portable and therefore can be transported to locations where it is required. Its operational season is not limited to the summer months as is a landfarm. It requires low maintenance. It can be used to pretreat waste sludge and reduce the amount of land required for a landfarm to treat an equivalent amount of waste. Currently we are actively seeking an engineering partner to form an alliance to investigate engineering aspects of the bioreactor and asses commercial applications of these bioreactor systems.

ACKNOWLEDGMENT

The support of ENERGY MINES AND RESOURCES CANADA (CANMET) for partial funding of some these studies is greatly appreciated. (DSS Contract #2344-8-9177/01-SQ, Scientific Authority: Dr. Ron McCready; and DSS Contract #2344-8-9263/01-SQ, Scientific Authority: Dr. John Donini).

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TABLE 1: COMPOSITION OF SOME SLUDGES GENERATED BY THE OIL INDUSTRY

SLUDGE TYPE	TONNES/YR	COMPOSITION (% WEIGHT)		
		OIL	WATER	SOLIDS
TANK BOTTOMS	100 - 3,000	30-60	40-70	SEDIMENT
DESALTER BOTTOMS	60 - 70	5 -40	30-85	10-40
API SEPARATOR BOTTOMS	100 - 20,000	10-40	50-90	5 -30

(From Reference 1)

TABLE 2: OIL LOSS IN THE PRINCE GEORGE BIOREACTOR

SAMPLING TIME	% OIL CONTENT	% OIL LOSS
START	28.4	0
4 WEEKS	28.4	0
8 WEEKS	25.4	10.5
8 MONTHS	7.2	75
11 MONTHS	7.3	74

TABLE 3. OIL CONTENT IN THE LLOYDMINSTER BIOREACTOR

SAMPLING TIME	API SEPARATOR SLUDGE		FILTER PRESS AID	
	OIL CONTENT (oil/solids)	OIL LOSS (%)	OIL CONTENT (oil/solids)	OIL LOSS (%)
Start	0.77	0	0.21	0
8 MONTHS	0.42	46	0.08	62
21 MONTHS	0.42	46	0.05	76

TABLE 4: ASPHALTENE CONTENT OF THE LLOYDMINSTER BIOREACTOR

SAMPLING TIME	% ASPHALTENE CONTENT	
	API SEPARATOR SLUDGE	FILTER PRESS AID
0 MONTHS	14	4
8 MONTHS	26	20

TABLE 5: PAH AND METALS CONTENT IN THE LLOYDMINSTER BIOREACTOR (mg/Kg or mg/L)

CONTAMINANT	SLUDGE	LEACHATE	PROVINCIAL LIMITS*
ARSENIC	<10	<0.1	500
CADMIUM	0.6	<0.002	100
CHROMIUM	10	0.006	500
LEAD	15	<0.03	500
MERCURY	0.015	<0.001	100
NICKEL	12	0.02	134
SELENIUM	<5	<0.05	100
THALLIUM	<10	<0.1	130
VANADIUM	20	0.004	300
PAH	ND	<1	

* These are provincial limits for a landfill.

TABLE 6: OIL DEGRADING MICROBES IN THE LYSIMETER COLUMNS

OIL ABSORBENT MATRIX	OIL DEGRADING MICROBES PER GRAM	
	TOP OF COLUMN	BOTTOM OF COLUMN
OCLANSORB (RATIO)+		
1:1	8.6×10^5	3.0×10^5
2:1	4.2×10^6	8.6×10^4
RECYCLED OCLANSORB (RATIO)+		
1:1	1.86×10^8	3.0×10^7
ANOTHER HEAT TREATED PEAT (RATIO)+		
1:1	$>4.6 \times 10^8$	ND*
4:1	$>4.6 \times 10^8$	ND*
FULLER'S EARTH (RATIO)+		
1:1	$>4.6 \times 10^8$	ND*

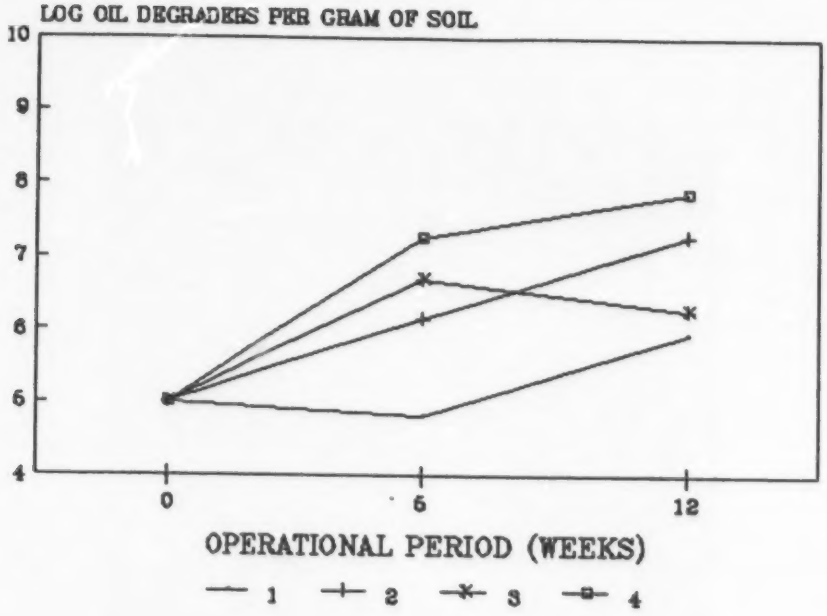
+ The ratio is the ratio of API Separator sludge to the matrix on a weight basis.
* ND - not determined.

TABLE 7: OIL LOSSES IN LYSIMETER COLUMNS

	START	END	% OIL LOSS
OCLANSORB			
1:1	208	172	17
2:1	365	286	22
3:1	244	239	2
4:1	181	173	4
ANOTHER HEAT TREATED PEAT			
1:1	108	92	15
4:1	370	336	9
FULLER'S EARTH			
1:1	250	243	3
RECYCLED OCLANSORB			
1:1	245	218	11
	(175)	(148)	(15)*

* The recycled Oclansorbd matrix from the bioreactor used in that particular lysimeter column already contained 69.8 grams of oil. This oil is non degradable as was demonstrated in shake flask studies. Correcting for the presence of this non degradable oil in the sample, the amount of degradation of the additional oil from the API Separator sludge is 15%.

FIGURE 1: INCIDENCE OF OIL DEGRADING MICROBES IN LANDFARM PLOTS AT PRINCE GEORGE



The experimental plots were treated in the following manner:

#1 received oily sludge only. The sludge was ploughed into the site.

#2 received oily sludge and fertilizer. These were spread on the soil before tilling.

#3 received oily sludge, fertilizer and raw peat (non heat treated) peat. The sludge was absorbed onto the peat before it was mixed with fertilizer and ploughed into the soil.

#4 received oily sludge, fertilizer and Oclansorb (heat treated peat). The sludge was absorbed onto the Oclansorb before it was mixed with fertilizer and ploughed into the soil.

FIGURE 2: ELECTRON MICROGRAPH OF OCLANSORB HEAT TREATED PEAT
(The material is filled with many small pores)

a) 70X Magnification

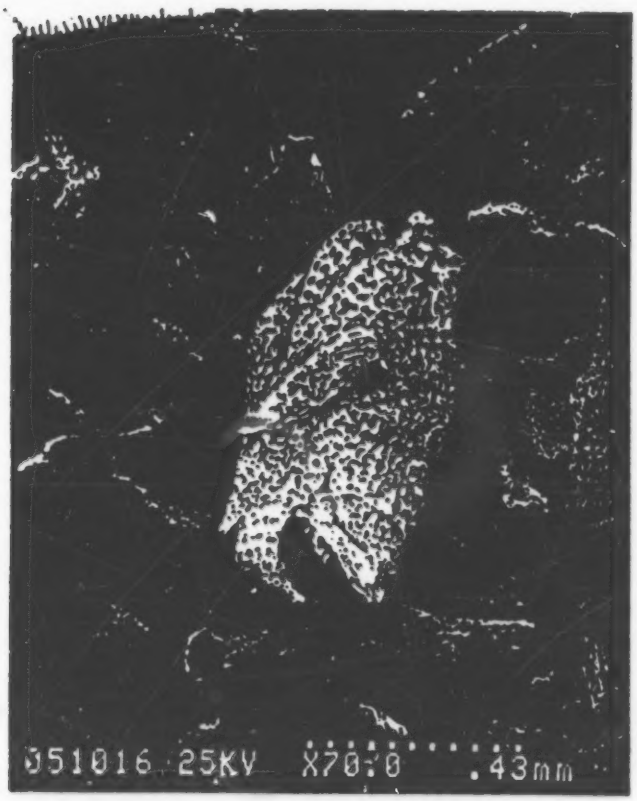
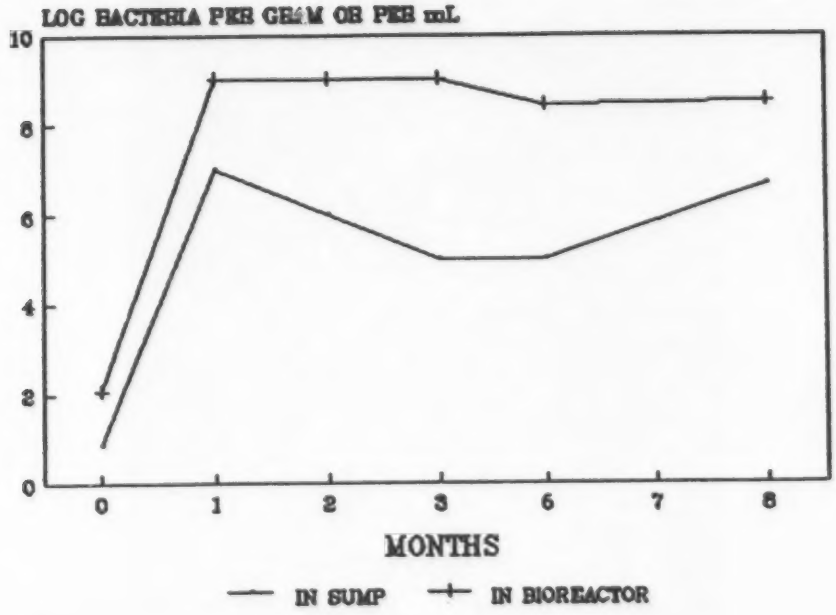


FIGURE 3: MICROBIAL ACTIVITY IN THE PRINCE GEORGE BIOREACTOR



Although the bioreactor was frozen at 2-3 months, the microbes associated with the matrix still remained viable.

FIGURE 4: BIOFILM OF HYDROCARBON DEGRADING MICROBES ON THE SURFACE OF OCLANSORB (The ropy convoluted structures make up the biofilm)

FIGURE 5: GAS CHROMATOGRAM OF THE OILY SLUDGE IN THE PRINCE GEORGE BIOREACTOR (The sludge consisted of saturate molecules with an average molecular size of C-30 - C-35)

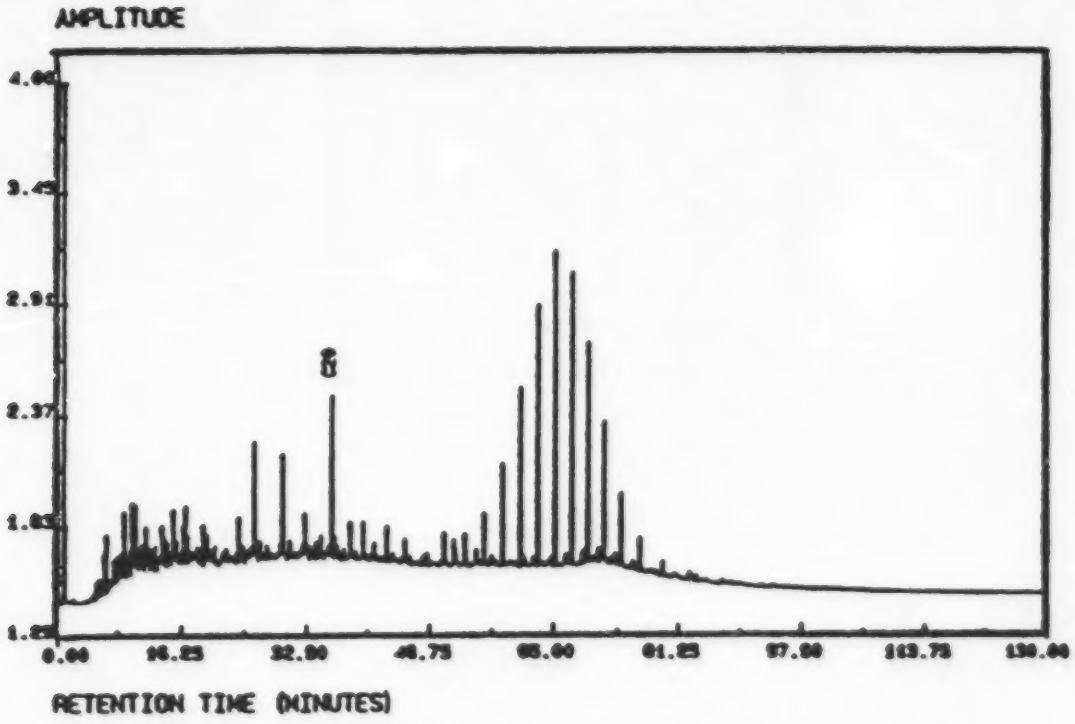
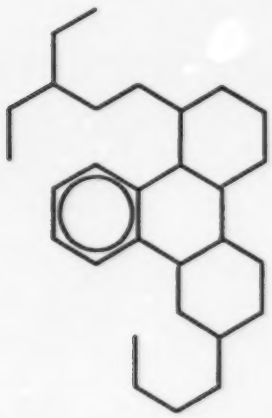
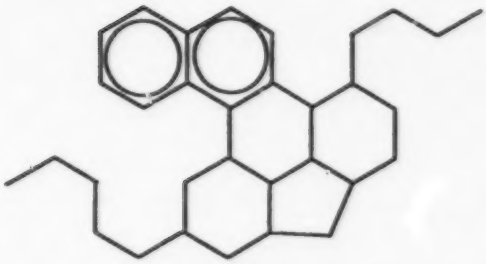


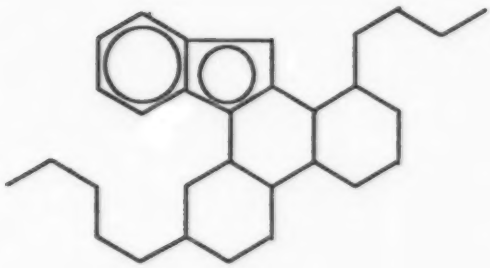
FIGURE 6: AVERAGE MOLECULAR STRUCTURES OF ALBERTA HEAVY CRUDES



Average structure for blended crude

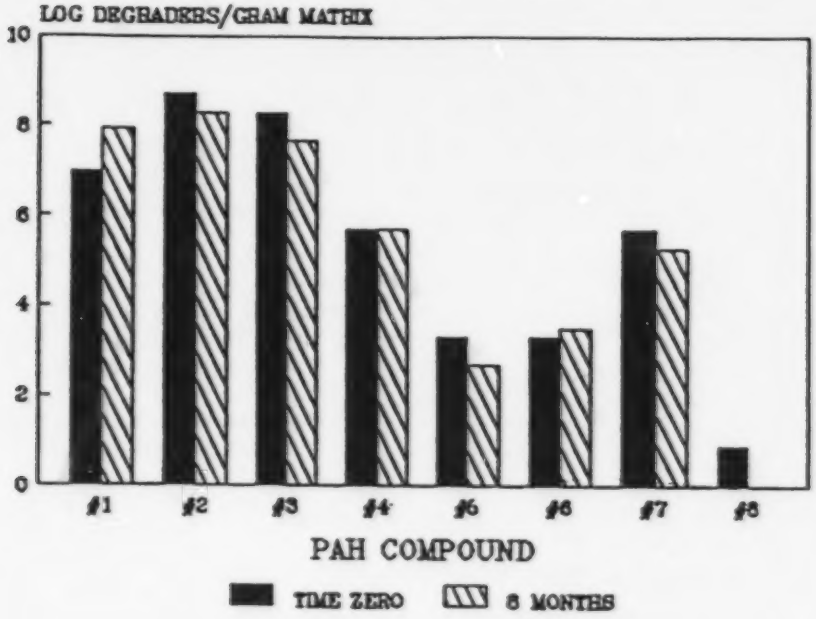


Average structure for native crude



Average structure for steamflood crude

FIGURE 7: HYDROCARBON DEGRADATION ABILITY OF OIL DEGRADING ORGANISMS IN LLOYDMINSTER BIOREACTOR OVER EIGHT MONTHS



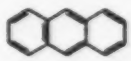
#1 - AMAULIGAK OIL

#2 - CATECHOL

#3 - TOLUENE

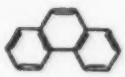
#4 - NAPHTHALENE

#5 - ANTHRACENE

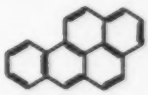


#6 - PYRENE

#7 - PHENANTHRENE



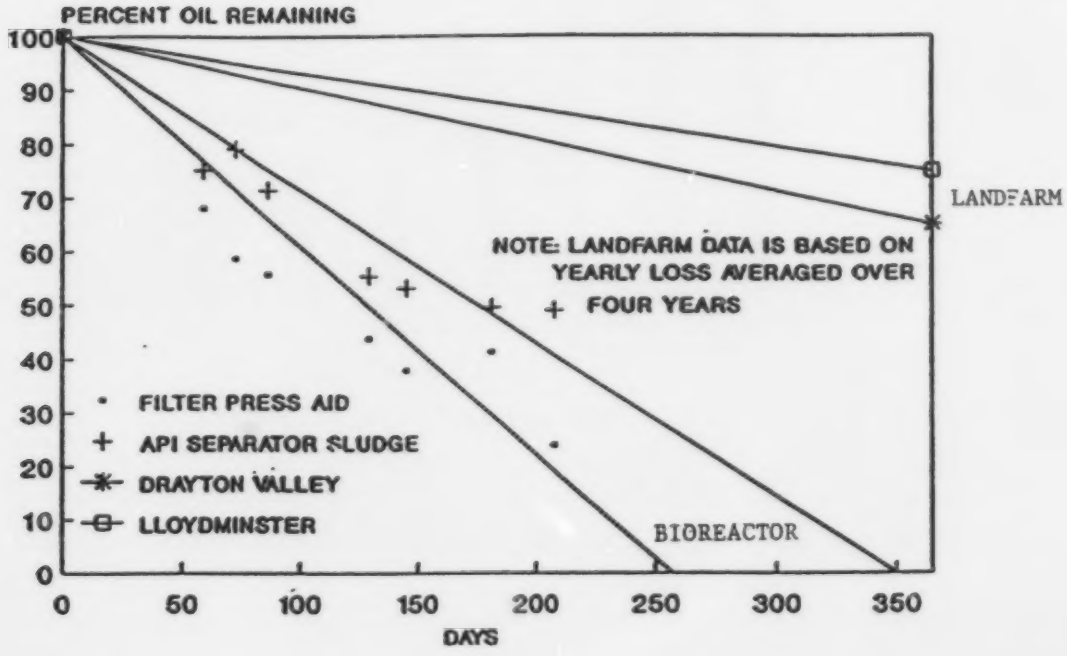
#8 - BENZO(A)PYRENE



#9 -ASPHALTENE *

* No growth was obtained on asphaltene as a carbon source.

FIGURE 8: COMPARISON OF OIL DEGRADATION RATES IN LLOYDMINSTER BIOREACTOR WITH RATES IN LANDFARMS AT LLOYDMINSTER AND AT DRAYTON VALLEY



MICROBIAL DEGRADATION OF THREE *n*-ALKYLTHIOPHENES

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ABSTRACT

The microbial metabolism of aliphatic and aromatic hydrocarbons which are abundant in petroleum and are commercially available as pure compounds has been studied extensively. In contrast, much less is known about the metabolism of sulfur-, nitrogen- or oxygen-containing compounds that are less abundant in petroleum.

Although hundreds of sulfur-containing compounds have been identified in petroleums, very few have been examined in biodegradation studies. Three *n*-alkylthiophenes, 2-hexadecyl-5-methylthiophene (I), 2-methyl-5-tridecylthiophene (II) and 2-butyl-5-tridecylthiophene (III), were synthesized and used as substrates in biodegradation studies. The products of their aerobic metabolism by pure and mixed bacterial cultures were identified. The long alkyl chains of these thiophenes were oxidized and in pure cultures of alkane-degrading bacteria, the major metabolites that accumulated in the medium were 5-methyl-2-thiopheneacetic acid from (I), 5-methyl-2-thiophenecarboxylic acid from (II), and 5-butyl-2-thiophenecarboxylic acid from (III). These transformations are consistent with the degradation of the alkyl side chains by the removal of acetate units through the beta-oxidation pathway. In the presence of Prudhoe Bay crude oil and a mixed culture of petroleum-degrading bacteria, the acid metabolites underwent further biotransformations.

DÉGRADATION MICROBIENNE DE TROIS *n*-ALKYLTHIOPHENES

RÉSUMÉ

La dégradation microbienne d'hydrocarbures aliphatiques et aromatiques, des espèces abondantes dans le pétrole et disponibles dans le commerce sous forme de composés purs, ont fait l'objet d'études exhaustives. Par contre, la dégradation des composés contenant du soufre, de l'azote et de l'oxygène, qui sont moins abondants dans le pétrole, est moins bien connue.

Même si des centaines de composés sulfurés ont été détectés dans les pétroles, très peu ont fait l'objet d'études de biodégradation. Nous avons synthétisé trois *n*-alkylthiophènes, soit le 2-hexadécyl-5-méthylthiophène (I), le 2-méthyl-5-tridécylthiophène (II) et le 2-butyl-5-tridécylthiophène (III), que nous avons ensuite utilisés comme substrats au cours d'études de biodégradation. Nous avons caractérisé leurs produits de dégradation en milieu aérobie par des cultures bactériennes pures et mixtes. Il y avait oxydation des longues chaînes alkyliques et, dans des cultures pures de bactéries capables de dégrader des alkanes, les principaux métabolites qui s'accumulaient dans le milieu étaient l'acide 5-méthyl-2-thiophèneacétique dans le cas de (I), l'acide 5-méthyl-2-triophènecarboxylique dans le cas de (II) et l'acide 5-butyl-2-thiophènecarboxylique dans le cas de (III). Ces transformations concordent avec la dégradation des chaînes alkyliques latérales comportant l'élimination des groupes acétates par oxydation ß. En présence de pétrole brut de Prudhoe Bay et d'une culture mixte de bactéries capables de dégrader le pétrole, les métabolites acides subissaient des biotransformations plus poussées.

INTRODUCTION

A recent review [7] demonstrated that relatively little is known about the microbial metabolism of organosulfur compounds in petroleum. Infromation available in the literature originates from three general areas of research. These include: (a) comparisons of the different organosulfur compounds present in petroleums that have undergone various amounts of biodegradation in their reservoirs; (b) laboratory and field studies that have addressed the fates of petroleum spilled in the environment; and (c) biodesulfurization studies in which microorganisms have been considered for the selective removal of organosulfur compounds from petroleum [13].

Hundreds of organic sulfur compounds have been identified in petroleum [4, 23, 26, 32, 34] and the structures of some of the general classes of organosulfur compounds are shown in Figure 1. Because so few petroleum-related organosulfur compounds are commercially available, less than twenty pure organosulfur compounds have been subjected to biodegradation studies [7]. Thus there is much to be learned about the fate of these compounds in petroleum-contaminated environments and about the biotransformations of these compounds in petroleum reservoirs. Progress in these areas can only be made through collaborations between chemists and microbiologists. Chemists synthesis organosulfur compounds that the microbiologists use as novel substrates for their microbial cultures.

Most studies on the biotransformations of individual petroleum-related organosulfur compounds have focussed on the condensed thiophenes including dibenzothiophene [17, 18, 20, 35], benzothiophene [2, 8, 29], and recently some alkylbenzothiophenes [28].

Investigations have shown that thiophene is resistant to biodegradation by aerobic microbial cultures whereas carboxythiophenes such as 2-thiophenecarboxylic, 3-thiophenecarboxylic, 2-thiopheneacetic and 5-methyl-2-thiophenecarboxylic acids are susceptible to microbial metabolism [1, 3, 6, 16]. Based on oxygen uptake experiments, Sagardía et al. [29] concluded that thiophene, 2-methylthiophene and 3-methylthiophene were degraded by a benzothiophene-adapted bacterial isolate. Fedorak et al. [9] observed that the side chains of two *n*-alkyltetrahydrothiophenes (*n*-alkylthiolanes) were attacked by *n*-alkane-degrading bacteria and fungi.

A variety of alkylthiophenes have been observed recently in immature sediments, oil shales and crude oils (e.g. ref. 31). These range from components with polycyclic and isoprenoidal to branched and linear carbon skeletons. Alkylthiophenes comprising a linear carbon skeleton, *n*-alkylthiophenes, have been observed in oil shales [19, 33] and crude oils [33]. Several classes have been identified extending from 2-alkyl-, 2-alkyl-5-methyl-, 2-alkyl-5-ethyl-, 2-alkyl-5-propyl- through

to the so-called "mid-chain" 2,5-dialkylthiophenes [33].

Three long-chain, *n*-alkylthiophenes (Figure 2) were synthesized and used in the present study. These were added individually to pure cultures of *n*-alkane-degrading bacteria to determine whether they were biodegradable and the major metabolites were subsequently identified. In addition, the *n*-alkylthiophenes were added to a mixed culture of petroleum-degrading bacteria with Prudhoe Bay crude oil to assess whether these compounds were degraded in the presence of petroleum and to determine whether the metabolites underwent further biotransformations in a mixed culture. Figure 3 summarizes the overall experimental procedures.

MATERIALS AND METHODS

Chemicals

The three *n*-alkylthiophenes used as substrates shown in Figure 2 were synthesized using methods outlined by Peakman and Kock-van Dalen [25]. 5-Methylthiophene-2-carboxylic acid, heptanoic acid and Raney nickel (activity W-2) were all purchased from Aldrich (Milwaukee, WI).

Bacterial Cultures and Culture Conditions

Six Gram-positive *n*-alkane-degrading bacteria isolated in our laboratory were used in this study. These strains were designated C1B, C1O, C1Y, C2, C3 (described in ref. 9) and SE (described in ref. 14). To prepare the inocula for liquid culture experiments, the isolates were grown on plate count agar (Difco, Detroit MI) at 28°C for 2 or 3 days. Then the cell growth was aseptically washed from the plate with 3 mM phosphate buffer (pH 7.2) and the resulting cell suspension was used as inoculum.

During the initial studies with compound I, the isolates were inoculated into 125-mL Erlenmeyer flasks containing 50 mL of liquid mineral medium [9] supplemented with trace metals solution [8] and 5 µL of the *n*-alkylthiophene as the sole carbon and energy source. In pure-culture studies with compounds II and III, 10 µL of the *n*-alkylthiophene were added to 50 mL of medium. All cultures were incubated for 14 days, unless otherwise noted, at 28°C with shaking to provide aeration.

The susceptibility of compound I to microbial attack was compared to the susceptibilities of *n*-hexadecane and pristane. Isolate C2 was inoculated into several 500-mL Erlenmeyer flasks that each contained 2 mg of *n*-hexadecane, 2 mg of pristane, 2 mg of compound I and 200 mL of mineral medium supplemented with trace metals. After various times of incubation at 28°C under shake-flask conditions, one of the

replicate cultures was analyzed to determine the proportions of *n*-hexadecane, pristane and compound I remaining compared to appropriate sterile controls.

A mixed bacterial culture, designated SLPB, was also used in this study. It was enriched from fuel-contaminated beach material from Shell Lake, Northwest Territories, Canada. This culture has been maintained since 1983 by monthly transfers to fresh medium containing Prudhoe Bay crude oil as the sole carbon source.

Two hundred millilitres of mineral medium supplemented with trace metals solution in 500-mL Erlenmeyer flasks containing 0.2-mL portions of Prudhoe Bay crude oil were inoculated with 20 mL of the mixed bacterial culture SLPB. Duplicate cultures were supplemented with 2 µL of compound I and two others were supplemented with 1 mg of 5-methyl-2-thiophenecarboxylic acid. A third pair of cultures received 2 µL of compound I with no crude oil supplement. Appropriate sterile controls were incubated with the active cultures. These were incubated on a rotory shaker at 28°C for 14 days prior to extraction for GC analysis.

Analytical Methods

After appropriate incubation times, the liquid cultures that received pure *n*-alkylthiophenes were acidified with sulfuric acid to pH <2 and extracted four times with 15 mL of methylene chloride to recover residual substrate and any metabolites that formed.

For routine monitoring for sulfur-containing metabolites, culture extracts were analyzed by capillary GC [8] using an instrument fitted with a flame ionization detector (FID) and a sulfur-specific, flame photometric detector (FPD) [10]. The DB-5 capillary column effluent was split so that the sample was analyzed by both detectors simultaneously. The oven temperature program was 90°C for 2 min, 4°/min to 250° for 16 min. Selected extracts with sulfur-containing metabolites were analyzed by electron impact GC-MS [12] at the analytical service facilities in the Chemistry Department at the University of Alberta. Methyl esters of the metabolites were prepared as outlined by Fedorak and Westlake [11].

Raney Nickel Desulfurization and Reduction

Raney nickel was washed with distilled water, 95% ethanol and anhydrous ethanol [15]. Culture extracts, free of methylene chloride, were refluxed for 4 h with 0.1 g of Raney nickel in anhydrous ethanol. After cooling, the nickel was extracted with dilute alkali [22]. Then the solution was made acidic with sulfuric acid and extracted with methylene chloride. The GC retention time of the carboxylic acid resulting from the Raney nickel reaction was matched with that

of the appropriate authentic standard.

RESULTS

Metabolism of 2-Hexadecyl-5-methylthiophene (Compound I) by Pure Cultures

Of the three *n*-alkylthiophenes, compound I was synthesized in the largest quantity and therefore its biodegradation was studied most intensively. Initial studies focussed on the Gram-positive isolate C2 grown on compound I. GC analysis of the extract from a 28-day-old culture showed three sulfur-containing compounds (Figure 4). The last peak to elute from the column was residual compound I. Metabolite A had the same retention time as a sample of the commercially available compound 5-methyl-2-thiophenecarboxylic acid. In addition, the mass spectrum of metabolite A (Figure 5) was virtually identical to that of 5-methyl-2-thiophenecarboxylic acid.

The mass spectrum of metabolite B showed a molecular ion at *m/z* 156 (Figure 5). The base peak was *m/z* 111 which corresponds to a loss of -COOH. A base peak at *m/z* 111 has been observed previously in the electron impact mass spectra of 2-alkyl-5-methylthiophenes resulting from the cleavage of the alkyl side chain beta to the thiophene ring [24, 34]. These results suggested that metabolite B was 5-methyl-2-thiopheneacetic acid.

To verify that the more abundant metabolite B was 5-methyl-2-thiopheneacetic acid, combined extracts from cultures of isolate SE grown on compound I were desulfurized and reduced with Raney nickel. This reaction was expected to yield heptanoic acid from 5-methyl-2-thiopheneacetic acid (Figure 6). Indeed, the product obtained from the Raney nickel reaction had the identical GC retention time as authentic heptanoic acid.

The other five *n*-alkane-degrading bacterial isolates were also able to use compound I as their sole carbon and energy source. Each isolate produced two sulfur-containing metabolites with the same GC retention times as those produced by isolate C2. GC-MS analyses of the extracts from isolates C1Y and SE showed that these metabolites were also 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic acid. The latter metabolite was more abundant than the former metabolite in culture extracts.

Figure 7 shows the results of the time course study with the three different substrates, *n*-hexadecane, pristane and compound I. As expected from the literature, *n*-hexadecane was degraded before pristane. *n*-Hexadecane was also degraded more quickly than compound I. However, compound I was consumed more quickly than pristane.

Metabolism of 2-Methyl-5-tridecylthiophene (Compound II) by Pure Cultures

Isolates SE and C2 were individually incubated for 14 days in liquid medium containing compound II as the sole carbon and energy source. GC analysis of the extract from isolate SE showed that it metabolized virtually all of the substrate leaving one predominant sulfur-containing metabolite. GC-MS analysis and comparison with an authentic standard showed that the product was 5-methyl-2-thiophenecarboxylic acid. There was also a trace of another metabolite that was 5-methyl-2-thiopheneacetic acid.

Analysis of the extract from isolate C2 showed two major and one minor sulfur-containing metabolites. GC-MS analysis demonstrated that one of the major metabolites was 5-methyl-2-thiophenecarboxylic acid and that the minor metabolite was 5-methyl-2-thiopheneacetic acid. The parent peak of the other major metabolite was the molecular ion at *m/z* 168 (Figure 8). Other observed ions and some proposed lost fragments were at *m/z* 151 (-OH), 123 (-COOH), and 97 (-CH=CHCOOH). The relative intensities of each of these ions were <40%. These results suggest that the metabolite was 5-methyl-2-thiophenepropenoic acid. The ion at *m/z* 111 was not abundant. This ion, which results from cleavage beta to the thiophene ring during electron impact mass spectrometry, is usually only abundant when a saturated alkyl chain is substituted onto the methyl-thiophene moiety.

The extract from isolate C2 grown on compound II was treated with methanol and sulfuric acid to produce the methyl esters of the carboxylic acid metabolites. GC-MS analysis showed the presence of a compound with a molecular ion *m/z* 182 which was consistent with the metabolite being 5-methyl-2-thiophenepropenoic acid. The base peak was at *m/z* 151 which corresponded to the loss of $-OCH_3$. The next most abundant ion was *m/z* 123 corresponding to the loss of $-COOCH_3$. Again the fragment ion at *m/z* 111 was rather weak which would be expected because of the position of the double bond in this metabolite.

Metabolism of 2-Butyl-5-tridecylthiophene (Compound III) by Pure Cultures

Isolates SE and C2 grew on compound III. GC analysis of the extract from isolate SE showed two sulfur-containing metabolites and the mass spectra of these metabolites are shown in Figure 9. The more abundant metabolite had a molecular ion at *m/z* 184 suggesting that the compound was 5-butyl-2-thiophenecarboxylic acid. The commonly observed cleavage beta to the thiophene ring that occurs during mass spectrometric analysis of alkyl-substituted thiophenes would

give the loss of $-CH_2CH_2CH_3$ and hence the ion at *m/z* 141 which can subsequently lose CO_2 yielding the ion at *m/z* 97.

The less abundant metabolite showed a molecular ion at *m/z* 198 (Figure 9) suggesting that the compound was 5-butyl-2-thiopheneacetic acid. Loss of $-CH_2CH_2CH_3$ via cleavage beta to the thiophene ring would give rise to the base peak at *m/z* 155 which can subsequently lose CO_2 to give the fragment at *m/z* 111. The fragment ion *m/z* 141 may have resulted from the loss of the entire butyl side chain by alpha cleavage.

Degradation of Compound I by the Mixed Culture SLPB

When the mixed culture SLPB was grown on compound I in the absence of crude oil, a small amount of 5-methyl-2-thiophenecarboxylic acid and a large amount of 5-methyl-2-thiopheneacetic acid remained in the medium after 14 days incubation. After a further 14 days of incubation, there was no detectable decrease in the amount of the more abundant metabolite.

Figure 10 shows the FID chromatograms of Prudhoe Bay crude oil supplemented with compound I extracted from a sterile control (top chromatogram) and a 14-day-old culture of SLPB. The mixed culture degraded the *n*-alkanes and isoprenoids, pristane and phytane, and compound I (bottom chromatogram). The GC chromatograms obtained from sulfur-specific FPD analysis of the extract from the SLPB culture that contained oil and compound I are shown in Figure 11. The top chromatogram is of the extract from the sterile control and the bottom chromatogram is of the extract from the mixed culture. The SLPB culture clearly removed all of compound I during the 14-day incubation period and there was no evidence of the accumulation of any sulfur-containing metabolites such as the 5-methyl-2-thiophenecarboxylic acid or 5-methyl-2-thiopheneacetic acid which were observed to accumulate in the pure cultures. In addition, many dibenzothiophenes were evident in the extract from the sterile control but were absent in the extract from the oil-degrading mixed culture.

These experiments demonstrated that in the presence of crude oil, the mixed bacterial culture could degrade compound I and transform the resulting metabolites, 5-methyl-2-thiophenecarboxylic acid and 5-methyl-2-thiopheneacetic acid. Clearly, some component of the crude oil is required to produce further transformations of the metabolites of compound I.

DISCUSSION

Metabolism of *n*-alkylthiophenes by Pure Cultures

The initial survey with compound I showed that each of the six *n*-alkane-degrading bacterial isolates grew on this

n-alkylthiophene. However, because there were only small amounts of compounds II and III available, only two of the isolates, SE and C2, were tested on these compounds. Both biodegraded these *n*-alkylthiophenes.

The results demonstrated that the long side chains of the *n*-alkylthiophenes were preferentially attacked and metabolized via a series of beta-oxidations. Metabolism of compound I, with a C_{16} side chain, yielded predominantly 5-methyl-2-thiopheneacetic acid which would result from the removal of seven acetate units by a series of beta-oxidations.

Metabolism of compounds II and III, with C_{13} side chains, yielded predominantly 5-methyl-2-thiophenecarboxylic acid and 5-butyl-2-thiophenecarboxylic acid, respectively. These would result from six beta-oxidations of the *n*-alkylthiophenes thereby shortening the side chains by twelve carbon atoms. During the beta-oxidation process, the action of an acyl dehydrogenase oxidizes a saturated intermediate yielding an unsaturated intermediate [21]. The detection of 5-methyl-2-thiophenepropenoic acid in the extract of isolate C2 grown on compound II is therefore consistent with the metabolism of this *n*-alkylthiophene via beta-oxidations. The enzymatic addition of water across the double bond in 5-methyl-2-thiophenepropenoic acid and subsequent removal of an acetate unit would yield 5-methyl-2-thiophenecarboxylic acid.

Interestingly, the butyl side chain of compound III remained unaltered indicating that it was too short to be attacked by these pure cultures of *n*-alkane-degrading bacteria. In studies with various *n*-alkylbenzenes [12], minimum side chain lengths of C_4, C_8 and C_9 were required for various *n*-alkane-degrading fungi to metabolize these hydrocarbons.

Small amounts of 5-methyl-2-thiophenecarboxylic acid and 5-butyl-2-thiopheneacetic acid were detected in cultures grown on compounds I and III, respectively. These products would not arise via beta-oxidations of the substrates but they might result from the alpha-oxidation (oxidative decarboxylation) [27] of one of the predominant metabolites. Alternatively, these may be the products of the beta-oxidations of trace contaminants in the preparations of compounds I and III. For example, GC-MS analysis of the preparation of compound I shows the presence of a contaminant with a molecular ion at *m/z* 308 and a base peak at *m/z* 111. These data suggest that the contaminant was 2-methyl-5-pentadecylthiophene which would yield 5-methyl-2-thiophenecarboxylic acid after a series of seven beta-oxidations.

The accumulations of carboxylic acids in the culture media observed during this study were similar to results of other investigations of the microbial metabolism of cyclic compounds

with long alkyl side chains. For example, Davis and Raymond [5] found that phenylacetic acid accumulated in cultures of various strains of *Nocardia* sp. grown on dodecylbenzene. Similarly, studies with *n*-alkane-degrading bacteria and fungi showed that the predominant metabolites from 2-undecyltetrahydrothiophene and 2-dodecyltetrahydrothiophene were 2-tetrahydrothiophenecarboxylic acid and 2-tetrahydrothiopheneacetic acid, respectively, and that these metabolites persisted in the culture media [9]. Other studies have shown that the resulting carboxylic acids were transient intermediates. These include work with *Nocardia salomonicolor* [30] and several fungi [12] grown on dodecylbenzene. In each of the above mentioned studies, the initial microbial attack was on the alkyl side chain rather than on the cyclic moiety.

Degradation of Compound I by the Mixed Culture SLPB

When added to Prudhoe Bay crude oil, compound I was metabolized by the oil-degrading mixed culture SLPB. Indeed, the presence of the crude oil enhanced the degradation of compound I because no carboxylic acid intermediates were detected in the extracts from the mixed culture supplemented with crude oil. However, the mixed culture did not transform the metabolites from compound I in the absence of crude oil in the medium.

The biotransformation products of the carboxylic acid intermediates of compound I have not been determined. However, several studies have shown that bacterial isolates can grow on various thiophene carboxylic acids. For example, Cripps [3] isolated a bacterium that could use 2-thiophenecarboxylic acid as its sole carbon, sulfur and energy source and Kanagawa and Kelly [16] isolated strains of *Rhodococcus* that used 2-thiophenecarboxylic acid, 5-methyl-2-thiophenecarboxylic acid and 2-thiopheneacetic acid as sole carbon and energy sources. Similarly, Evans and Venables [6] isolated a *Vibrio* species that could grow on 2-thiophenecarboxylic acid and 2-thiopheneacetic acid. Oxygen uptake experiments showed that resting cells of the *Vibrio* species were able to oxidize several thiophene derivatives including 2-methylthiophene, 3-thiophenecarboxylic acid, and 3-thiopheneacetic acid. Whether the carboxylic acid intermediates of the three *n*-alkylthiophenes served as growth substrates for some of the bacteria in the SLPB mixed culture or whether these intermediates were cometabolized to products that were not detected by our analytical method remains to be determined.

CONCLUSIONS

This work showed that each of the three model *n*-alkylthiophenes studied were readily metabolized by *n*-alkane-degrading bacteria that removed the side chain via beta-oxidations leaving carboxylic acid intermediates.

Compound I was less susceptible to microbial attack than *n*-hexadecane but more susceptible than pristane.

When added to a crude oil, compound I was also metabolized by a mixed culture and the carboxylic acid intermediates were further transformed to unidentified products. Although *n*-alkylthiophenes are usually minor components of petroleum, they are quite susceptible to microbial metabolism and therefore should not accumulate in petroleum-contaminated environments.

ACKNOWLEDGEMENTS

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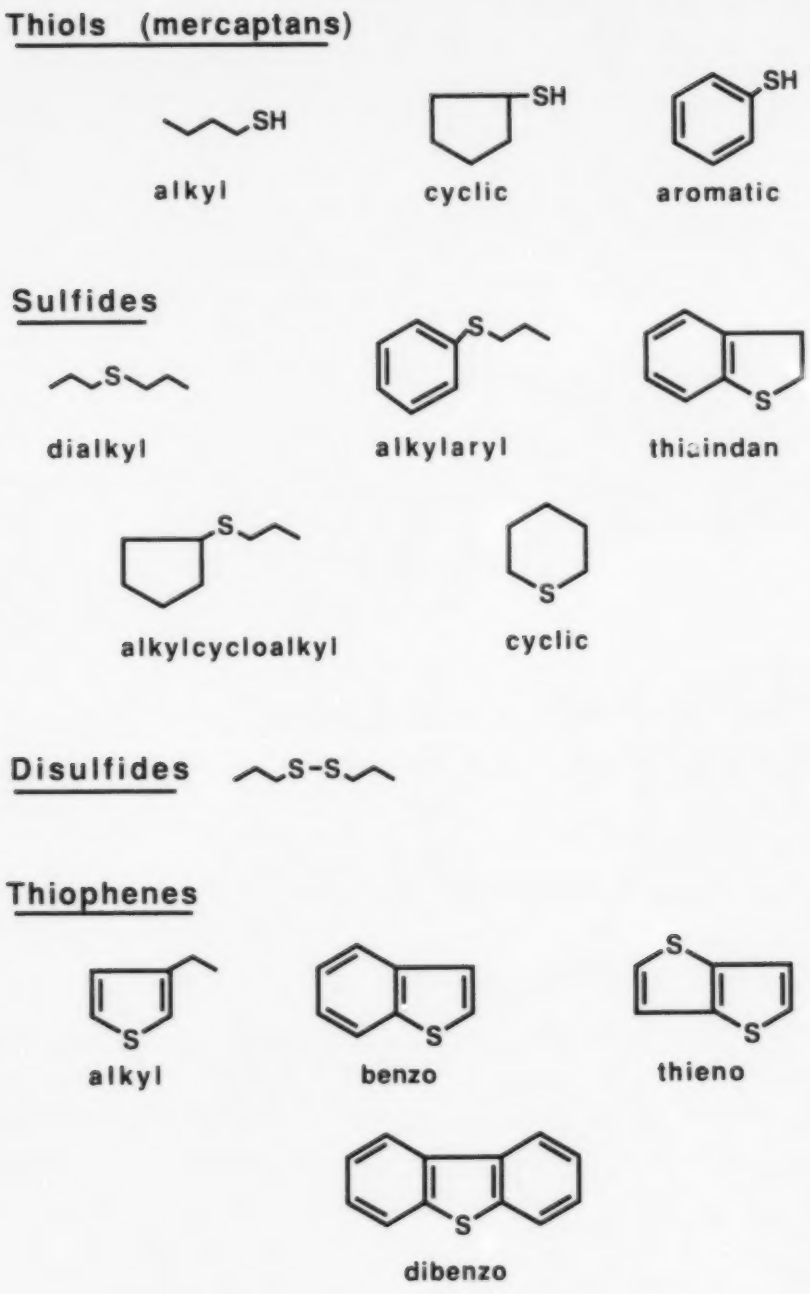
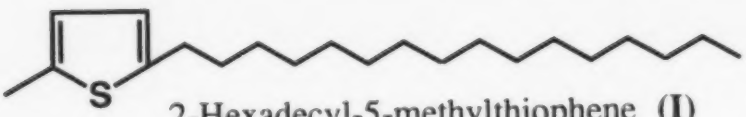
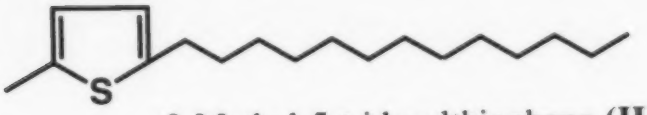


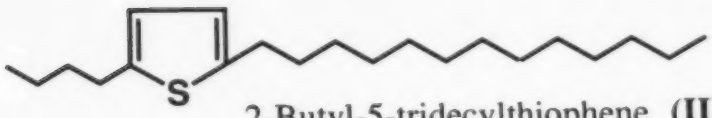
Figure 1. CLASSES OF ORGANOSULFUR COMPOUNDS IDENTIFIED IN PETROLEUM. AFTER [26].



2-Hexadecyl-5-methylthiophene **(I)**



2-Methyl-5-tridecylthiophene **(II)**



2-Butyl-5-tridecylthiophene **(III)**

Figure 2. STRUCTURES OF THE *n*-ALKYLTHIOPHENES USED IN THIS STUDY. THE ABBREVIATION FOR EACH COMPOUND IS GIVEN IN BRACKETS.

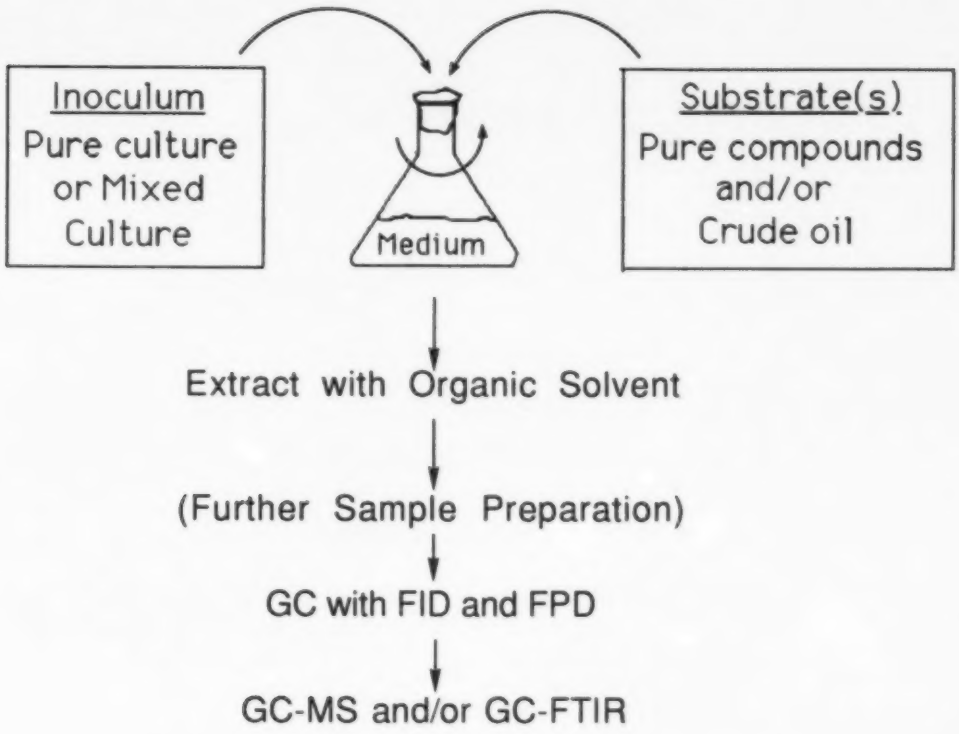


Figure 3. OVERVIEW OF THE METHODS USED TO STUDY THE BIODEGRADATION OF THREE *n*-ALKYLTHIOPHENES FOUND IN PETROLEUM.

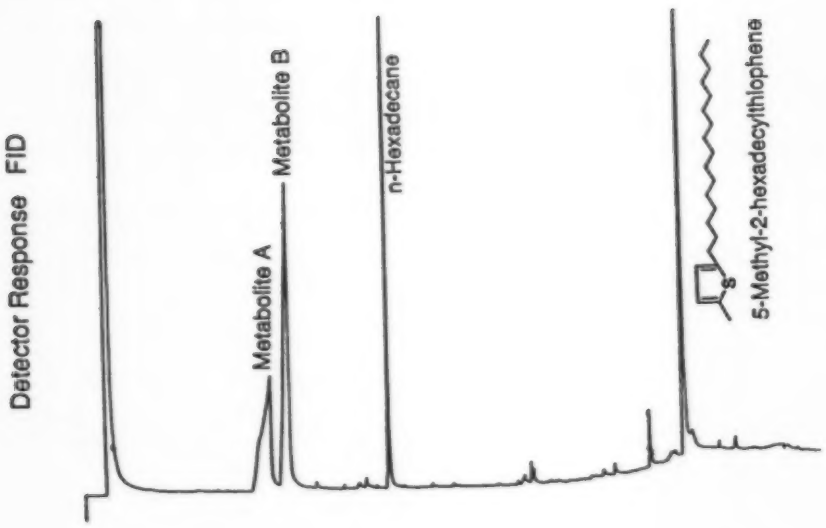


Figure 4. GC ANALYSIS OF THE METHYLENE CHLORIDE EXTRACT OF ISOLATE C2 GROWN ON COMPOUND I. *n*-HEXADECANE WAS ADDED AS A GC REFERENCE STANDARD.

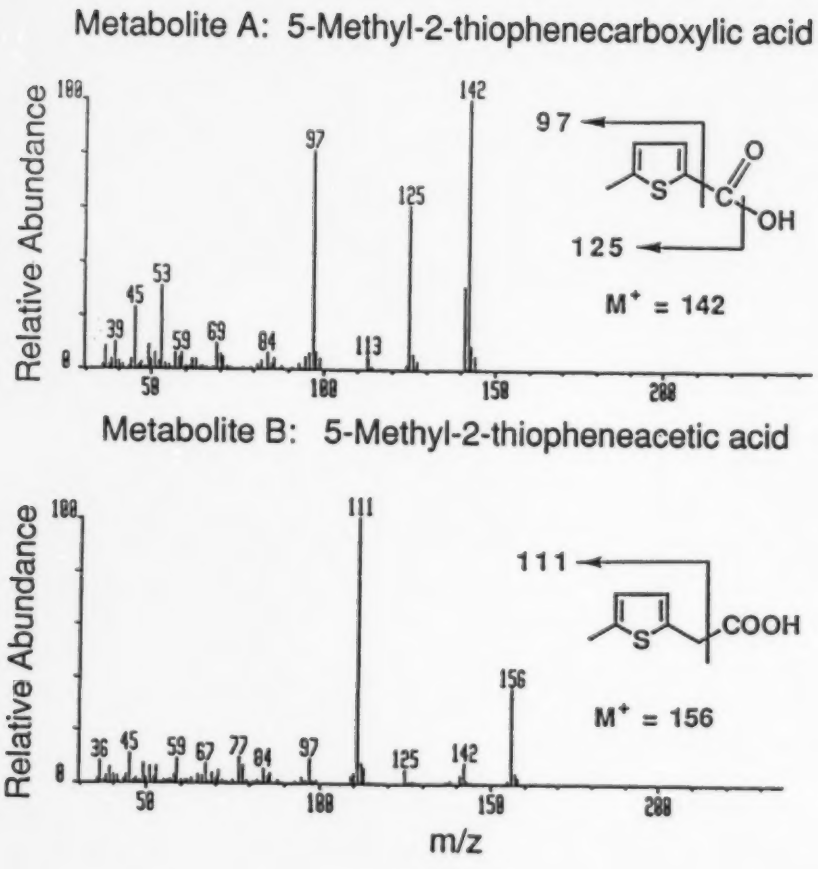
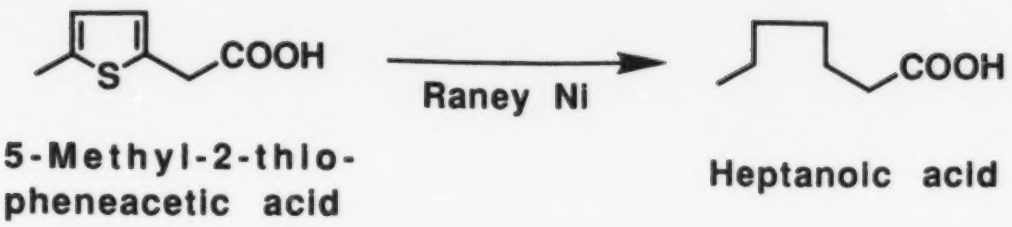


Figure 5. MASS SPECTRA OBTAINED FROM THE GC-MS ANALYSIS OF THE EXTRACT FROM ISOLATE C2 GROWN ON COMPOUND I.



5-Methyl-2-thiopheneacetic acid

Heptanoic acid

Figure 6. ANTICIPATED REACTION OF THE RANEY NICKEL REDUCTION AND DESULFURIZATION OF METABOLITE B THAT WAS PROPOSED TO BE 5-METHYL-2-THIOPHENEACETIC ACID.

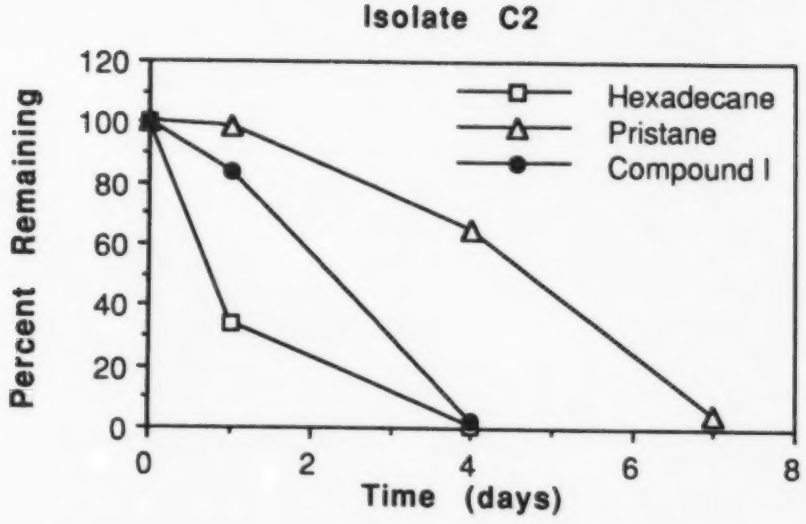


Figure 7. COMPARISONS OF THE RELATIVE SUSCEPTIBILITIES OF HEXADECANE, PRISTANE AND COMPOUND I TO BIODEGRADATION BY BACTERIAL ISOLATE C2.

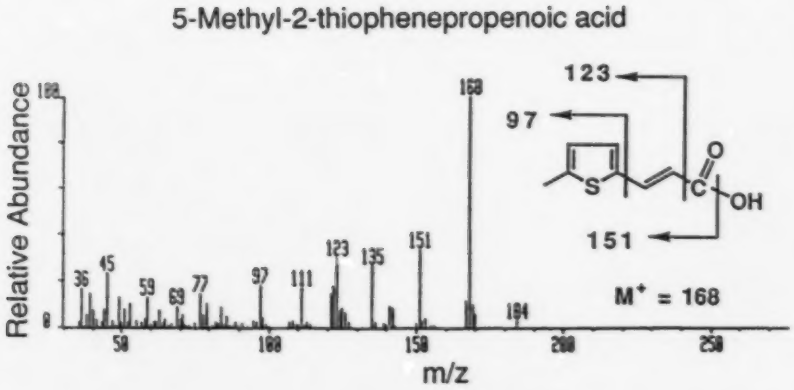


Figure 8. MASS SPECTRUM OF ONE OF THE METABOLITES OBSERVED IN THE GC-MS ANALYSIS OF THE EXTRACT FROM ISOLATE C2 GROWN ON COMPOUND II.

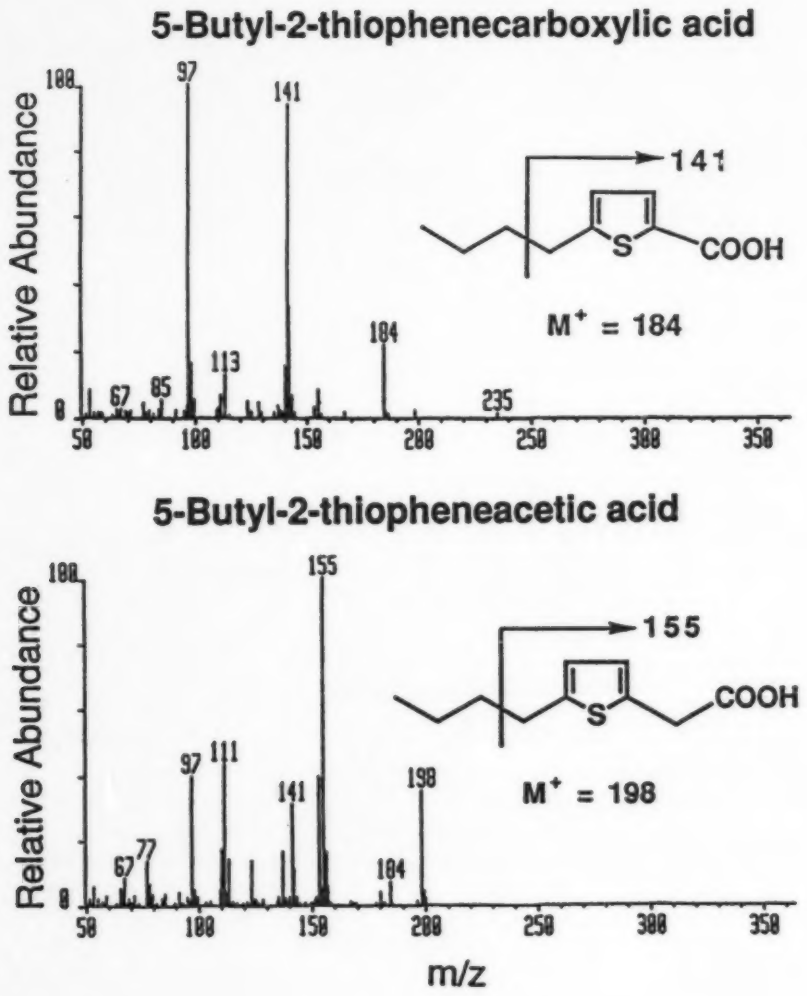


Figure 9. MASS SPECTRA OBTAINED FROM THE GC-MS ANALYSIS OF THE EXTRACT FROM ISOLATE SE GROWN ON COMPOUND III.

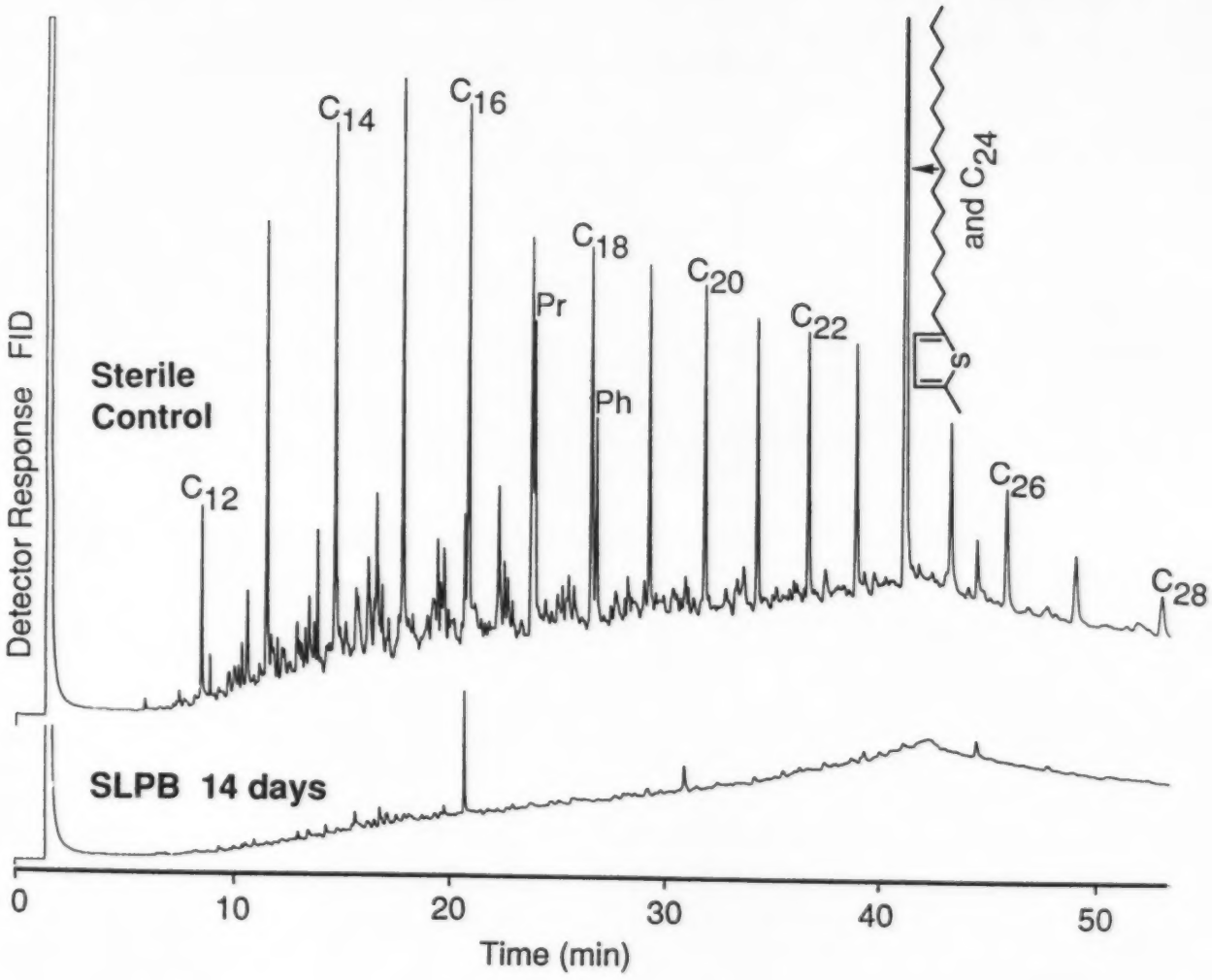


Figure 10. FID CHROMATOGRAMS OF EXTRACTS FROM CULTURES AFTER 14 DAYS INCUBATION WITH PRUDHOE BAY CRUDE OIL SUPPLEMENTED WITH COMPOUND I.

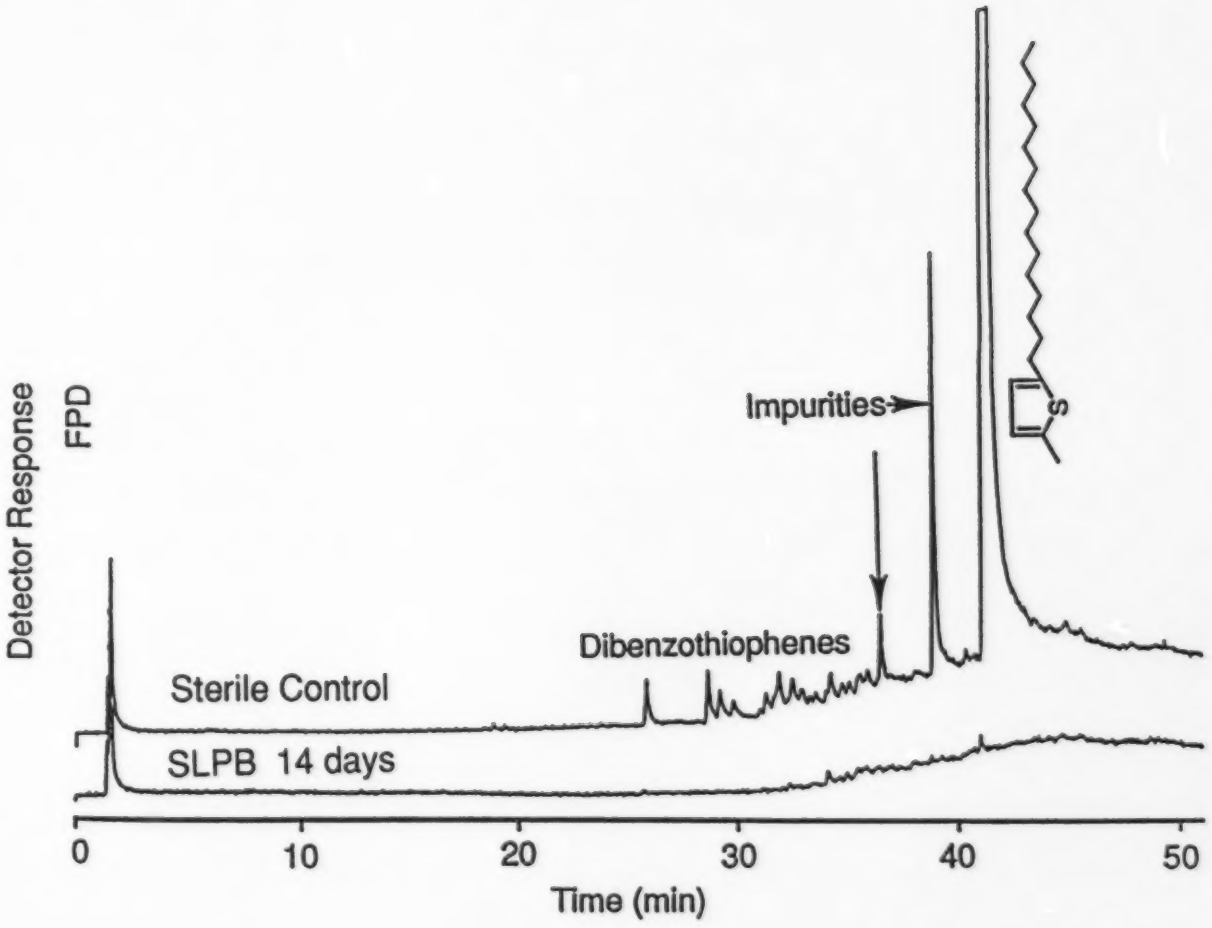


Figure 11. FPD CHROMATOGRAMS OF EXTRACTS FROM CULTURES AFTER 14 DAYS INCUBATION WITH PRUDHOE BAY CRUDE OIL SUPPLEMENTED WITH COMPOUND I.